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# PRACTICAL CLINICAL BIOCHEMISTRY

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BY

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# PREFACE

The rapid development of clinical biochemistry has been an outstanding feature of medicine during the past twenty to thirty years. The present book is a survey of the whole field of this subject from the standpoint of workers in hospital laboratories. It is hoped that it will appeal particularly to registrars training in clinical pathology, to hospital biochemists, and to laboratory technicians. The book should be especially useful to technicians studying for the examination in Chemical Pathological Technique of the Institute of Medical Laboratory Technology. As the tests described are also used in many laboratories not directly concerned with diagnosis and treatment, so far as possible the needs of workers in medical research laboratories have also been borne in mind.

While the purpose of the book is essentially practical, it was felt that summaries of the findings in health and disease would add considerably to its value. Although these are necessarily brief they are provided to obviate frequent reference to larger works on the interpretation of biochemical tests. No claim is made that these replace entirely such books a few examples of which are given in the short bibliography on p. 520.

My debts to other authors are many. In a rapidly developing subject such as this much is owed to the various journals which publish papers on biochemical methods. Full references to these are given. Books on practical methods which I have found valuable are also listed in the bibliography already referred to.

I wish gratefully to acknowledge permission to use material by the following: *Acta medica Scandinavica*, for material from Supplement 128 (1942) by Dr. H. O. Lagerlöf; *American Journal of Physiology* for Table XX; Messrs. Baird and Tatlock for Fig. 6; Bayer Products Ltd. for Fig. 4; British Drug Houses Ltd. for the list of primary standards on p. 513; Messrs. J. and A. Churchill Ltd. for normal values for hæmoglobin from "Practical Hæmatology," by J. V. Dacie on p. 401; Evans Electroselenium for Figs. 56 and 57; Harvard University Press for Fig. 58 adapted from "Chemical Anatomy, Physiology and Pathology of Extracellular Fluid," by J. L. Gamble; Ilford Ltd. for Figs. 10 and 11; *The Journal of Clinical Endocrinology* and Dr. S. Soskin for Fig. 14; *The Journal of Clinical Investigation* for Fig. 55; *The Lancet* for Figs. 18, 19 and 36; *The Lancet* and Dr. J. D. Robertson for Table XXI; Messrs. H. K. Lewis and Co. Ltd. for material on p. 245 from the *British Journal of Experimental Pathology*; Messrs. E. S. Livingstone for the table of Standard Weights on p. 518 from "A Textbook of Dietetics," by L. S. P. Davidson and I. A. Anderson; Dr. G. Lusk for Table XXIII; The Josiah Macy Jr. Foundation for material from the booklet "Copper Sulphate Method for Measuring Specific Gravities of Whole Blood and Plasma," by Phillips *et al.*; Messrs.

May and Baker Ltd. for material on pp. 502–505 from their booklet "The Estimation of Sulphonamides"; Shandon Scientific Company for Fig. 37; Dr. M. Somogyi for Table XVI; Dr. J. H. van de Kamer for Fig. 54; Drs. D. D. van Slyke and G. E. Cullen for Table XIX; Messrs. Williams and Wilkins for material on Indicators and Buffers on pp. 514–516 from "The Determination of Hydrogen Ions," by W. M. Clark (published in Great Britain by Baillière, Tindall and Cox Ltd.); Year Book Publishers, Chicago, for Table XXIX from "Methods in Medical Research," Vol. 2, edited by J. H. Comroe.

I should like especially to express my thanks to my wife who did nearly all the diagrams and formulæ, and to several of my friends and colleagues from whose help I have benefited considerably. Dr. J. E. Keneh has read the whole of the proofs whilst Dr. H. T. Howat, Dr. S. W. Stanbury, Mr. M. Bell and Mr. J. E. Southall have each read sections. I have received constant encouragement and advice from Dr. R. W. Fairbrother. For any errors either of omission or commission which still remain the author must accept sole responsibility.

Finally I should like to thank my publishers for their unfailing consideration and patience during the period in which the book was being written and published.

H. V.

*October*, 1953.

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*CHAPTER I*

# INTRODUCTORY. COLLECTION OF SPECIMENS AND SOME GENERAL TECHNIQUES

## BLOOD

### Collection of Blood Specimens

Capillary or venous blood is used for almost all determinations made on blood.

**Capillary blood** is most frequently obtained from a finger or thumb. The most convenient place is on the thumb about half a centimetre from the side of the nail. The tip of a finger is also used. The finger or thumb is first cleaned with ether or methylated spirit, and is then pricked smartly with a sharp surgical needle. A better flow of blood is obtained when the hand is warm, and if the arm is first allowed to hang limply and is then gently swung backwards and forwards for a short time. After making the prick, a tourniquet of thin narrow tubing is wrapped round from the base of the thumb outwards, and sufficient pressure exerted to force out the blood, which is allowed to run directly into a suitable pipette held inclined at an angle slightly downwards from the horizontal. When the technician is familiar with the technique, the pipette, in many cases, fills easily and quickly. If, however, blood ceases to flow before the calibration on the pipette is reached, it is better not to exert undue pressure, but to unwrap the tourniquet and allow the arm to swing loosely for a moment or two to restore the circulation of the thumb. The tourniquet is then rewrapped and more blood collected. From two to three minutes is available before the blood begins to clot.

Special attention should be paid to ensuring that the pipettes used are clean and dry, otherwise they will not fill easily. Pipettes are cleaned in chromic acid, washed free from acid with water, and dried by drawing a small quantity of acetone through by means of a suction pump. To complete the evaporation of the acetone, the pipettes can be warmed slightly by passing them quickly through a Bunsen flame. A pipette with a straight bore (Fig. 1) is more satisfactory than the type with a bulb. When using the latter, a small bubble of air sometimes remains in the bulb when the blood enters the neck of the pipette.

This method is usually employed when it is desired to collect 0·1 or 0·2 ml. The blood is then placed immediately into some other solution so that no anticoagulant is required. In the hands of a skilled technician as much as 0·5 to 1·0 ml. can sometimes be obtained. In this case the blood can be allowed to drop into a small tube containing a little anticoagulant.

Some workers prefer to use the lobe of an ear. The technique is

rather more difficult to acquire. Again amounts up to 0·2 ml. are collected in a pipette, larger volumes into a small tube containing anticoagulant. Amounts up to 3 ml. can be obtained by allowing blood to accumulate repeatedly and then expressing into such a tube. This is particularly valuable when it is difficult to obtain venous blood. In the case of infants the heel is used. The ankle is grasped to congest the foot and a prick made in the heel.

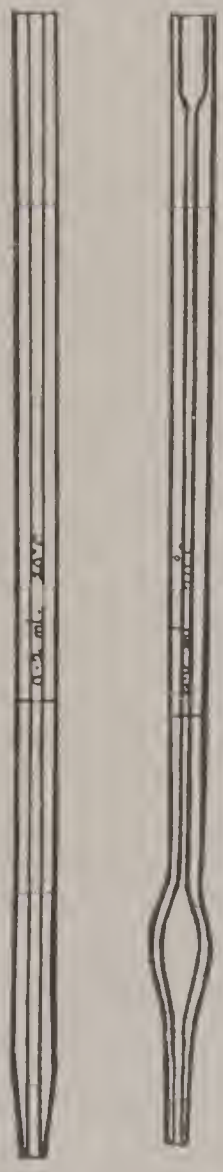

Fig. 1. Blood pipettes.

These methods for collecting blood are widely used. Many estimations have been adapted to use 0·2 ml. of capillary blood. However, this small volume is only suitable for determinations which can be done on whole blood.

Capillary blood is similar in composition to arterial blood.

**Venous blood** must be collected for a large number of estimations. If serum is required the blood is allowed to clot, but is mixed with an anticoagulant if whole blood or plasma is to be used. While the blood may be taken from any prominent vein, a vein on the front of the elbow or forearm is almost universally employed. The arm should be warm. This improves the circulation and distends the vein. The surface of the skin is cleaned by rubbing with alcohol or ether. The arm is extended and a rubber tourniquet firmly applied a few inches above the elbow. This should not obliterate the arterial pulse at the wrist. A well sharpened sterile hypodermic needle fixed on to a syringe of appropriate capacity—usually 10 ml., though if a number of tests are to be carried out on a single specimen, a 20 ml. syringe may be required—is inserted into the vein, which can be held steady by the thumb of the other hand of the operator. When the needle enters the vein the plunger is withdrawn slightly. If blood appears the tourniquet is released, since as little congestion as possible should be present. When the desired amount of blood has been drawn into the syringe, a small pad of cotton wool soaked with spirit or ether, is placed on the arm where the needle was inserted, and the needle withdrawn. This pad is held on for a few minutes until bleeding stops. The needle is removed from the syringe and the blood transferred to an appropriate container, using the minimum amount of pressure. Immediately after use the needle and syringe are washed out with cold water to remove any blood remaining.

**Arterial blood** is rarely examined. It has been taken for blood gas determinations and when studying the arterial-venous ratio for the

blood sugar. It is most commonly obtained by inserting a needle into the radial, brachial, or femoral artery, usually under a local anæsthetic.

## Cleaning and Sterilizing Syringes

The piston is removed and the barrel washed thoroughly with water, using a test tube brush. The piston is also rubbed with the brush, special care being taken to see that the back of the piston ring is kept clean. To dry the syringe a little methylated spirit or acetone is drawn in and then expelled as completely as possible. The piston after drying is smeared with a little liquid paraffin as lubricant, then replaced in the barrel and the needle attached. The needle is covered with a small metal or glass tube so as to protect the sharp end, and the whole then placed in a container, the mouth of which is well plugged with cotton wool to prevent movement of the syringe. A glass test tube can be used but a metal container is better. Maw & Co., of London, supply aluminium ones which are very satisfactory (see Fig. 2). If these are used, after plugging with cotton wool, the cap is screwed on and the outfit sterilized in an autoclave or dry air oven. It is a good practice to stick a label STERILE IF LABEL UNBROKEN over the cap, so that this cannot be unscrewed without breaking the label. This method of sterilizing can only be used for all-glass syringes, or for record type syringes with ceramic pistons. If syringes have metal pistons the barrel and piston should be disconnected and sterilized separately.

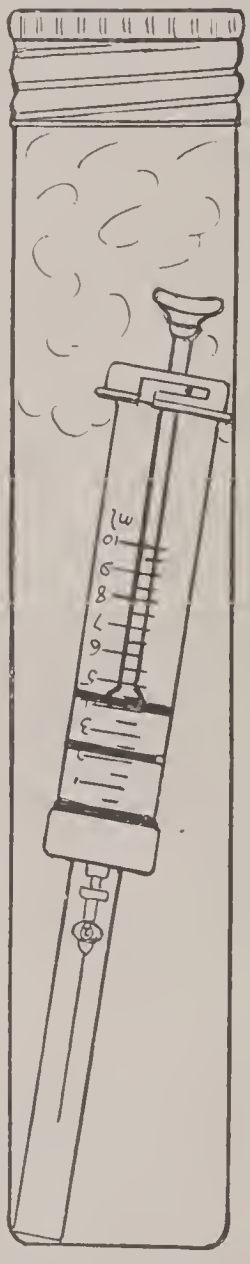

FIG. 2. Container for sterilizing Syringes.

All types of syringe can be sterilized by means of hot oil from an oil bath. The hot oil (liquid paraffin is suitable) at 130° C. to 140° C. is drawn into and forced out of the syringe several times over a period of a half to one minute. This method has been widely used in the past, but has been criticized on the grounds that sterilization may not be effected. This may be so if the oil is not kept long enough in the syringe, as may be the case when it is only drawn in once or twice and is quickly ejected.

Sterilization by placing the parts of the syringe in boiling water (containing a pinch of sodium bicarbonate to prevent rusting) for twenty minutes, has been used, but is not recommended. A little water left in the syringe may cause some hæmolysis.

## Sharpening Needles

Efficient sharpening of the needle is most important. A white Arkansas stone (Fig. 3), such as is used by joiners for sharpening gouges, has been found satisfactory. The stone has two edges with different bevels. Needles are sharpened on these edges, which have curves suitable for those of the cutting edges of different sizes of needles. The use of xylol as a lubricant will prevent glazing of the surface of the stone. The needle, held firmly near to the point by the thumb and first finger, is drawn backwards and forwards a distance of 2 to 3 cm. along the edge chosen, and never allowed to leave the surface of the edge while this is being done. When the sharpening is finished the slightly rough outer edges of the bevel can be smoothed by gently rubbing them on the flat surface of the stone.

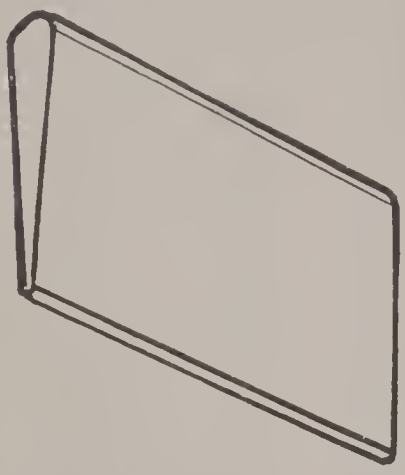

Fig. 3. Stone for sharpening Needles.

## Venules

For use outside hospitals, and for those who only wish to take a specimen of blood occasionally, venules (Fig. 4) are convenient. The sterile needle is supplied covered with a small glass tube, which is broken by cutting with the small file included, and the needle then inserted into a vein in the usual way. By manipulating the tube

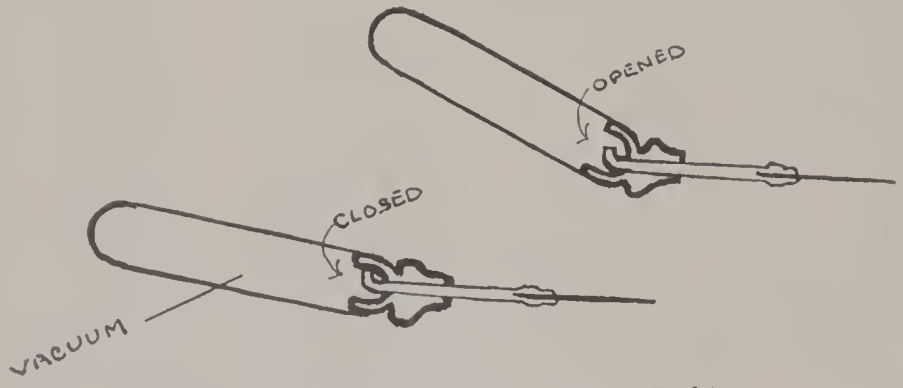


Fig. 4. Venules (Bayer Products, Ltd.).

as shown in the diagram, the valve in the rubber stopper is opened, and the blood is drawn into the tube by the negative pressure inside. The blood is mixed with the anticoagulant by gentle shaking or by rotating the tube between the palms of the hands. Venules can be obtained, either with or without anticoagulant, for a variety of purposes from Bayer Products Ltd., London. Full instructions are given with the tubes.

## Avoiding Hæmolysis

When plasma or serum is to be used it is essential to avoid hæmolysis. Even when hæmolysis is not noticeable on inspection, in many specimens spectroscopic examination will show the presence of bands of oxyhæmoglobin. Provided these are faint, their presence may be ignored, but any appreciable degree of hæmolysis, certainly visible hæmolysis, makes the serum or plasma unsuitable for some determinations. It is easier to obtain serum almost free from hæmolysis than plasma, since the anticoagulant present is an additional factor which may produce lysis of the cells.

Certain precautions can be taken to avoid hæmolysis. The syringe and needle must be dry. As lubricant a little liquid paraffin may be used if desired. When taking blood, a minimum amount of constriction is applied to the arm. The blood should flow slowly and steadily into the syringe, and after removing the needle, should be expelled slowly into the tube used. Mechanical breakdown of red cells is thus avoided as far as possible. Tubes into which the blood is placed should be clean and dry. The anticoagulant should be mixed with the blood by gentle rotation. Excessive amounts of anticoagulant should not be used. Separation of plasma should be done by centrifuging at low to moderate speeds. If the plasma is not required immediately, the cells can be allowed to sediment out, the supernatant fluid removed and finally cleared of any remaining cells by centrifuging. In the case of serum, the blood may be allowed to clot in a centrifuge tube, cooled for some time in the refrigerator, and then centrifuged; or the blood is allowed to stand until the clot has contracted, when the serum can be poured off, and centrifuged. If it is necessary to loosen the clot, this should be done as gently as possible with a thin, clean, dry glass rod. Another useful practice is to put the blood into a wider tube than usual and allow clotting to take place with the tube slanted. When the blood is firmly clotted, the tube is stood upright so that the serum then drains off.

## Anticoagulants

Heparin is the most satisfactory anticoagulant since it produces no change in the blood. Nevertheless, because they are much cheaper and more easily obtained, oxalates and citrate have been most used. It is important not to use more of these salts than is necessary, otherwise appreciable changes in the distribution of water between cells and plasma may result, and they may interfere with some determinations.

**Potassium or Sodium Oxalate.** These act by precipitating the calcium. Potassium oxalate has been most commonly used of the oxalates since it is the most soluble. Ten to 20 mg. per 10 ml. blood are required to prevent clotting, but 20 to 30 mg. should be used. Technicians making up tubes should become familiar with this amount. The salt is used finely powdered. Peters and Van Slyke (1932) recommend preparing a 30 per cent. solution of neutral

potassium oxalate, recrystallized if necessary, and adjusted to a *p*H of 7·4 ± 0·2 by adding potassium hydroxide or oxalic acid solution. An appropriate amount, 0·1 ml. for 10 ml. of blood, is measured into the tube which is to hold the blood, is spread on the walls as a film by rotating the tube, and then dried in a stream of air. The blood then mixes easily and rapidly with the oxalate and very little further shaking is necessary. This is specially useful for specimens to be taken under oil.

**Sodium Citrate.** Citrate does not precipitate the calcium but converts it into a non-ionized form. Nevertheless citrated plasma is not as satisfactory as serum for the determination of calcium. About 30 mg. of sodium citrate per 10 ml. of blood will prevent clotting. About twice this amount should be used.

**Ammonium Oxalate (3 parts) + Potassium Oxalate (2 parts)** has been used (Heller and Paul, 1934) in certain hæmatological investigations since it does not affect the red cell volume. One mg. per ml. has been suggested, but 2 mg. per ml. may be necessary to ensure that clotting does not occur. Ammonium oxalate should not be included in the anticoagulant for any determination in which ammonia is being produced, for example, in urease urea estimations, Kjeldahl protein and non-protein nitrogen determinations.

**Heparin** is effective when about 2 mg. per 10 ml. of blood is used. It should be used when it is important to ensure that no change takes place in the blood.

**Sodium Fluoride** also acts as an anticoagulant, but larger amounts are required than of either citrate or oxalate, so that it is chiefly used as a preservative, when it is usually mixed with potassium oxalate (see p. 7). It is not recommended as an anticoagulant for as much as 100 mg. per 10 ml. of blood is necessary. Fluoride is an enzyme poison.

## Changes in Blood on Keeping

The following are among the changes which may take place in blood when it is kept.

1. Loss of carbon dioxide. Since the carbon dioxide content of plasma is higher than that of air, some is lost from the plasma to the atmosphere. This leads to the passage of carbon dioxide from the cells into the plasma, formation of carbon dioxide from bicarbonate in the cells, and to compensate for this, passage of bicarbonate from plasma to cells. Even so, the bicarbonate content of the cells is reduced so that chloride passes from cells to plasma to keep the ratio of bicarbonate to chloride the same in both cells and plasma. In order to prevent loss of carbon dioxide and this series of changes, the blood is collected under liquid paraffin and the plasma separated immediately.

The simplest procedure is to take the blood into a syringe containing a little liquid paraffin but no air, and transfer the blood immediately to a centrifuge tube containing liquid paraffin and oxalate. The end of the needle is placed under the liquid paraffin in this tube and the blood gently forced out. It is mixed with the anti-

coagulant by rotating the tube between the palms of the hands. The tube is then filled with liquid paraffin, corked and centrifuged. Air should be excluded during centrifuging since carbon dioxide may be lost when the blood is shaken. The plasma should be removed from the cells immediately after centrifuging, and care taken not to include any oil when pipetting from this plasma.

For work involving a high degree of accuracy Peters and Van Slyke (1932) collect the blood over mercury, and describe an apparatus by which this can be done.

2. Conversion of glucose to lactic acid, glycolysis (see p. 29).

3. Increase in plasma inorganic phosphate due to formation from ester phosphate present in the cells (see p. 303). To avoid this, serum or plasma is separated within a short time from taking the blood.

4. Formation of ammonia from nitrogenous substances of which urea is the chief, may occur in blood which has been contaminated with bacteria. This may interfere with the estimation of potassium (see p. 350). The blood should be kept sterile or in a refrigerator.

5. Passage of substances through the red cell envelope. This is important, for example, in the case of potassium which is present in much greater concentration in the cells than in the plasma. Serum or plasma should be separated shortly after taking the blood.

6. Conversion of pyruvate into lactate (see p. 418). The blood should be mixed immediately with the protein precipitant or preserved as described on p. 419.

If blood is to be kept it should be collected under aseptic conditions. As preservative a mixture of potassium oxalate and sodium fluoride in the proportions of three parts to one is used. Sodium fluoride mixed with thymol (20 : 1) has also been employed. John (1926) used 20 mg. of this mixture per 10 ml. of blood but other workers have used more. Roe *et al.* (1927) used up to 100 mg. of sodium fluoride per 10 ml. when the blood was sterile. It could then be kept for several days and still be examined for sugar and non-protein nitrogen. More is required if the blood is not kept sterile. Since sodium fluoride inhibits enzyme activity it interferes with the determination of urea using urease.

In general, serum or plasma should be separated as soon as possible after taking the blood. All blood specimens should be kept in a refrigerator.

## Type of Blood to be Used

The reasons for the choice of whole blood, serum or plasma for a determination will be discussed when each substance is being considered. There are, however, some general points of importance.

1. When the substance to be estimated is evenly distributed between cells and plasma, it does not usually matter which is used. It may be more convenient to use whole blood, particularly if the test can be done using 0·2 ml. of blood.

2. There are a number of blood constituents, for example glucose, uric acid, creatinine, in the determination of which, substances

present in the cells behave in the same way as the substance which is being estimated, thus giving higher values than the true ones. Use of serum or plasma avoids interference from these. Whole blood placed into isotonic sodium sulphate has also been used for this purpose (see pp. 29, 33).

3. In some cases variations in plasma level of the substance are significant, for example, for plasma chlorides.

4. Since it is easier to obtain serum free from hæmolysis there is a good case for the use of serum rather than plasma when it is necessary to separate off the cells. Plasma, however, must be used when the separation has to be made immediately. Thus plasma is used in the determination of bicarbonate and chloride. To summarize: use

**Whole Blood.** Glucose, urea, non-protein nitrogen, sulphonamides.

**Serum.** Uric acid, creatinine, creatine, calcium, sodium, potassium, cholesterol, bilirubin, acid and alkaline phosphatase, amylase, lipase, albumin and globulin, inorganic phosphorus, carotenes, vitamin A, amino-acids, bromide, thiocyanate, salicylate.

**Plasma.** Chloride, bicarbonate (alkali reserve), *p*H, fibrinogen, ascorbic acid.

## Protein Precipitants

The first stage in the majority of determinations made on blood is to remove proteins. For this purpose many substances are used, chiefly heavy metal salts, acids, and organic substances. Tungstic acid and trichloracetic acid are most often employed.

**Tungstic acid** (Folin and Wu, 1919). The reagents used are 10 per cent. sodium tungstate ($Na_2WO_4$, $2H_2O$) and 2/3 N sulphuric acid. The tungstate solution should not be acid to phenolphthalein, or more alkaline than to need 0·4 ml. of N/10 hydrochloric acid to make 10 ml. neutral to this indicator. To prepare 2/3 N sulphuric acid 20 ml. of concentrated acid is diluted to 1 litre, standardized against a known sodium hydroxide solution, and adjusted if necessary. This acid liberates the whole of the tungstic acid from an equal volume of the tungstate, and practically all this is taken down by the protein precipitate giving a filtrate which is just to the acid side of neutral, having a *p*H approximately 6·6.

Folin's technique is to mix 1 volume of whole blood with 7 volumes of water, add 1 volume of tungstate, mix, and then add 1 volume of the sulphuric acid slowly, drop by drop, shaking well during the addition. The mixture is then allowed to stand for ten minutes before filtering. With whole blood the final colour should be dark brown. If this colour is not produced it is necessary to add a little more acid. For this purpose, 10 per cent. sulphuric acid is added one drop at a time, shaking well after each addition. Care should be taken not to add more acid than is required. If, on filtering, the first portion of the filtrate is cloudy, it should be refiltered through the same filter paper. When using plasma or serum, 1 volume is taken with 8 volumes of water and a $\frac{1}{2}$ volume each of tungstate and acid.

Haden (1923) modified this procedure. He mixed 1 volume of

blood with 8 volumes of N/12 sulphuric acid, added 1 volume of the tungstate with constant shaking, allowed to stand for a few minutes, and filtered. Van Slyke and Hawkins (1928) added 1 volume of blood to 9 volumes of a mixture containing one part of 10 per cent. sodium tungstate and eight parts of N/12 sulphuric acid. This is convenient but the mixture does not keep well. It should not be kept for more than a fortnight, and should be discarded if any precipitate forms.

Bacterial action can take place in these filtrates so that estimations should be carried out within a few hours unless a preservative is added. Enzyme action is also possible, so that these filtrates can be used for such methods as the urease method for determining blood urea.

Sodium tungstate has also been used with copper sulphate, the copper tungstate formed acting as the protein precipitant.

**Trichloracetic Acid.** This gives an acid filtrate, which is valuable when an acid medium is necessary to keep in solution the substance, for example phosphate, which is being estimated. Trichloracetic acid usually gives a greater volume of filtrate from the same amount of blood, and filters more quickly than does the tungstic acid mixture. Ten per cent. trichloracetic acid may be used, 9 volumes being added slowly with constant shaking to 1 volume of blood. Alternatively, one volume of blood can be diluted with 4 volumes of water and 5 volumes of 20 per cent. trichloracetic acid added slowly. Proteins are precipitated completely and do not take any of the non-protein nitrogen with them.

**Alkaline Zinc Salts.** Somogyi (1930) used two solutions, 10 per cent. zinc sulphate ($ZnSO_4$, $7H_2O$) and 0·5 N sodium hydroxide. Ten ml. of the zinc sulphate, diluted to about 50 ml. with water, should require between 10·8 and 11·2 ml. of the hydroxide to become just alkaline to phenolphthalein. To precipitate the proteins, 1 volume of blood is diluted with 7 volumes of water and 1 volume of zinc sulphate solution added. After mixing, 1 volume of the hydroxide is added slowly with constant shaking. The mixture is allowed to stand for a few minutes and then filtered. As with the tungstate method, if plasma or serum is used, 1 volume is diluted with 8 volumes of water and a half volume of both zinc sulphate and sodium hydroxide added.

*Cadmium sulphate* has also been used. Somogyi (1945) does not find this any improvement, but has substituted *barium hydroxide* for sodium hydroxide. Whereas zinc sulphate and sodium hydroxide satisfactorily deproteinize whole blood, they may fail to do so completely with plasma. Barium hydroxide and zinc sulphate completely deproteinize whole blood, plasma, and serum. Other advantages claimed for this method are that it does not introduce any salts into the filtrate, and that it precipitates anticoagulants such as oxalate and fluoride, which if present in too large amounts sometimes interfere with the removal of protein. The solutions used are 0·3 N barium hydroxide and 5 per cent. zinc sulphate. An example is given on p. 36 in Somogyi's blood sugar method.

These methods are considered by Somogyi to precipitate along with the proteins, some, if not most, of the material which reduces the solutions used in estimating blood glucose (see p. 29). However, other methods have been used to achieve this. Folin (1930) suggested the use of unlaked blood in order to get a filtrate which did not contain certain non-glucose reducing substances. Since these are mostly present in the cells Folin has added 1 volume of the blood to 8 volumes of a solution containing 15 grams of anhydrous sodium sulphate (or 34 grams of the decahydrate) and 6 grams of sodium tungstate ($Na_2WO_4$, $2H_2O$) per litre. In this solution the cells are not laked. After standing five minutes, 1 volume of N/3 sulphuric acid is added slowly, whilst mixing gently. The cell envelopes are carried down with the protein precipitate, leaving only the diffusible components in solution. This principle has been used in several later methods, for example by King and his co-workers (see p. 38).

**Use of Alcohol, Ether, Acetone.** In a few methods, use of these organic substances is convenient, particularly if it is desired to extract some blood constituent into the organic solvent. Thus, for example, alcohol-ether mixtures are used to precipitate proteins and extract fats and cholesterol, whilst alcohol is used in the estimation of bilirubin, and acetone in icteric index measurements.

## Measuring Blood

Although ordinary pipettes can be used to measure plasma or serum, they cannot be used with whole blood because of its greater viscosity, without introducing appreciable error. For this reason, pipettes used for measuring whole blood should be calibrated to contain the amount of blood which they are designed to measure, and the blood should be washed out of the pipette. With pipettes measuring small amounts of blood, such as 0·1 or 0·2 ml., the blood is usually delivered into a measured amount of a solution and the pipette washed out by sucking the liquid up into it, and blowing it out, until all the blood is washed out. If the blood can be run into one part of the fluid and another part be used for washing out the pipette before the whole is mixed, it is even less likely that a small amount of blood will be left behind in the pipette.

Small blood pipettes should be calibrated by weighing the amount of mercury they can contain. The specific gravity of mercury is 13·57 at 10° C., 13·56 at 14° C., 13·55 at 18° C. Because of the difference in the shape of the meniscus a slightly smaller volume of mercury is weighed, than the volume for which the pipette is being calibrated. This varies with the diameter of the bore at the graduation mark. Examples are: 0·0005 ml. for a bore of 1·0 mm., 0·0015 ml. for 1·5 mm., 0·0025 ml. for 2·0 mm., and 0·0040 ml. for 2·5 mm. A correction curve is given by Peters and Van Slyke (1932). Once the required amount of mercury has been weighed out in a small beaker, it can be used to calibrate a whole series of pipettes by drawing up the mercury or transferring it from one pipette to another. The mercury should be clean and dry.

When using pipettes delivering larger volumes, the blood should

be washed out by passing water, or some solution used in the test, through the pipette. In calibrating such pipettes a suitable volume of water is weighed, the pipette filled to a provisional mark from this, and the remaining water reweighed. The mark is adjusted and the process repeated until an accurate calibration is achieved.

In pipetting whole blood, care should be taken to see that the cells are evenly distributed through the plasma. This is particularly important if the blood is being used for the determination of some constituent which is present in different concentrations in the cells and in the plasma.

## URINE

### Collection of Urine Specimens

Single specimens of urine are used for ward examinations and for most qualitative tests, but for quantitative work twenty-four-hour specimens are best employed, except when collecting specimens as part of tests such as urea clearance and concentration tests. Since great variations in concentration occur during the day, the daily output of a substance must usually be determined if significance is to be attached to the result of urine analyses. For this purpose it is most convenient to collect the specimen from one morning to the next. At some suitable time, such as 8 a.m., the patient empties the bladder and the urine is discarded. All specimens passed during the day, and during the following night are saved and the specimen obtained by emptying the bladder at the same time the following morning is added to them. If the patient wishes to defæcate the bladder should be emptied just before this is done so as to avoid loss of urine. The collection of twenty-four-hour specimens is subject to an appreciable risk that the specimen will be incomplete, a point which should always be borne in mind in interpreting results.

### Changes on Keeping

The chief changes which urine may undergo on keeping are due to bacterial action. Urine for chemical examination is not usually collected aseptically so it is not sterile at the time of collection. However, if the urine is passed into, and kept in, clean, well-washed receptacles and containers, little change will take place in a twenty-four-hour specimen by the time it is received in the laboratory, particularly if it has been kept in a cool place and is covered or stoppered. It is important that such specimens should be sent to the laboratory as soon as possible after they have been collected.

The most important effect of bacteria is on the urea, which is converted into ammonium carbonate. The ammoniacal odour of grossly contaminated urine is well known. Such urines are obviously unsuitable for the determination of urea, ammonia, *p*H, and, since ammonia may be lost to the air, of total nitrogen. Micro-organisms, both bacteria and yeasts, may also act on glucose. If the urine becomes alkaline, phosphates may be precipitated. On standing, uric acid and urates are deposited since they are less soluble in cool

urine. Before carrying out estimations, any deposit must be well mixed with the urine before sampling. Uric acid and urates can be redissolved by moderate warming, phosphates by adding a little acid.

Among other changes which may occur are the oxidation of urobilinogen to urobilin, and the rapid oxidation of ascorbic acid.

## Preservatives for Urine

If urines have to be kept it may be necessary to prevent some of the changes described above. The choice of preservative is often influenced by the purpose for which the urine is being collected.

As a rule the use of acid is satisfactory. Ten ml. of concentrated *hydrochloric acid* is adequate for a twenty-four-hour specimen. Such urines are suitable in particular for the determination of urea, ammonia, and total nitrogen. Uric acid may be precipitated, so the deposit must be well mixed before a sample is taken.

Chloroform, toluene, petrol ether, thymol, and formalin have been used, but have disadvantages. *Toluene* and *petrol ether* form a thin layer on the surface and this tends to contaminate pipettes. This can be partly overcome by placing the urine in a separating funnel and running off the lower layer of urine. Moreover, these substances only prevent further surface contamination with bacteria, they do not stop the growth of bacteria already introduced during the passage and collection of urine. *Chloroform* is more satisfactory in both these respects, but less so in other ways. It may interfere with some tests such as the detection of glucose, since it reduces Fehling's solution. The chloroform can, however, be boiled off. Since it settles to the bottom chloroform mixes with any deposit. Sufficient chloroform is used to give a saturated solution in the urine. *Thymol* is also used in an amount which will give a saturated solution. *Formalin*, 3 to 4 drops to 100 ml., can be used. If more is added it may give positive results with tests for glucose. To preserve urine for determination of ascorbic acid, either 10 per cent. concentration with *acetic acid*, or 5 per cent. with *metaphosphoric acid* is used.

It is satisfactory in most cases to use specimens collected with reasonable attention to cleanliness and to keeping the urine cool. Otherwise acid should be used where possible. A few crystals of thymol or a few ml. of toluene may be used in other cases. Any further points will be dealt with under the examinations in question.

## Volume of Urine

The daily output of urine on an average diet and normal fluid intake is between 1,200 and 1,500 ml., but it may vary widely both physiologically and pathologically. Whilst it is greatly influenced by the amount of fluids taken, the type of diet also has a considerable effect. Thus a high protein diet increases the output of urine because of the diuretic effect of the increased quantity of urea formed. If the temperature of the surroundings is high, volume is diminished; exercise has a similar effect. The minimum volume of urine needed to remove the daily waste products in health is about 500 ml.

An increased daily urine volume—*polyuria*—is found in a number

of conditions. The largest volume of urine excreted is in diabetes insipidus, when 10 to 20 litres and over may be passed, the condition being due to a lack of posterior pituitary hormone. In diabetes mellitus up to 5 to 6 litres may be excreted daily (see p. 72). In the later stages of chronic glomerulonephritis there is some degree of polyuria of the order of 2 to 3 litres daily. An increased amount may be excreted in some nervous disorders.

A reduced urine volume—*oliguria*—is found in fevers, in acute nephritis, in the early stages of chronic glomerulonephritis, in acute liver conditions, in diarrhœa, and in cardiac failure.

Total suppression of urine—*anuria*—may occur for a period in certain conditions, such as shock, acute nephritis, mercury poisoning, incompatible blood transfusion, in sulphonamide therapy, and bilateral renal stones.

The ratio of day urine (8 a.m. to 8 p.m.) to night urine (8 p.m. to 8 a.m.) is at least 2 : 1 and sometimes 3 : 1 or more in health. In renal disease this ratio is reduced, and may even be reversed.

## Specific Gravity of Urine

**Measurement of Specific Gravity.** 1. *Use of Urinometer.* In the simplest method a urinometer, shown in Fig. 5, is used. Weighted with mercury, this floats in the urine so that the calibration which corresponds to the surface level of the urine is read. The lower the specific gravity the further the urinometer sinks. Since the instrument is usually calibrated at 15° C., it is necessary to correct for temperature. This is done by adding 0·001 for every 3° C. above 15° C. and subtracting 0·001 for every 3° C. under 15° C. Make sure the urinometer clears the sides of the vessel. It is advisable to check the zero of a new instrument, and to compare a few results with those obtained by weighing.

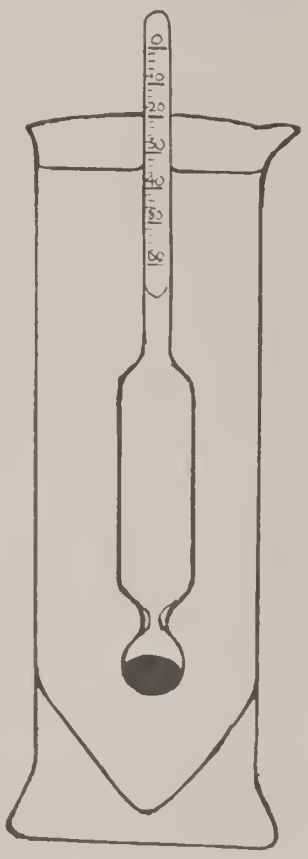

Fig. 5. Urinometer.

2. *Weighing.* For smaller amounts of urine it may be necessary to use weighing. In such cases a specific gravity bottle of suitable size may be used or a pipette taken and the weight of equal volumes of urine and water ascertained. The ratio of these gives the specific gravity.

3. In cases such as ureteric catheterization where only a very small amount of urine is available, the specific gravity can be determined as follows. Take a mixture of two liquids such as benzene (S.G. 0·879) and chloroform (S.G. 1·504) or of xylol (S.G. 0·863) and carbon tetrachloride (S.G. 1·58) and allow a drop of urine to fall into it. Then by adding one or other liquid according to whether the drop falls or not, a mixture is finally obtained in which the drop of urine stays suspended.

The specific gravity of this mixture is then measured by means of a urinometer, standardized for such mixtures.

4. *Specific Gravity Beads.* A set of glass beads can be used for ascertaining the specific gravity of urine. The one which neither sinks nor floats is found and the specific gravity marked on this is read. Sets of these beads can be obtained from Messrs. T. O. Blake Ltd., 40 Hatton Garden, London, E.C.1.

INTERPRETATION

Under ordinary conditions the specific gravity of a twenty-four-hour specimen is in the region of 1·020, ranging from about 1·016 to 1·025. Individual specimens of urine vary widely in their specific gravity. After drinking large amounts of water, specific gravity may be as low as 1·002, whilst when perspiring heavily it may reach and exceed 1·040. Variations in the specific gravity of normal urine mainly reflect changes in the amount of urea, sodium chloride and phosphates present. The specific gravity of pathological urines may be influenced in addition by the presence of glucose and albumin. The high specific gravity of the urine in diabetes mellitus is due to the high concentration of glucose. To allow for the increase in the specific gravity due to albumin, 0·003 is subtracted from the observed specific gravity for each per cent. of albumin present.

## FÆCES

Fæces are composed of

1. Products of digestion which have not been absorbed.
2. Undigested and undigestible parts of the food, such as starch granules, cellulose fibres, partly digested muscle fibres.
3. Substances from the secretions into the intestine, such as enzymes, changed bile pigments, mucus.
4. Cells from the intestinal wall, occasional red blood cells and leucocytes, all of which may be increased in illness.
5. Products derived from the foodstuffs, such as indole, skatole, gases, fatty acids, resulting from the action of bacteria.
6. Bacteria.
7. Water.
8. Foreign bodies such as concretions, parasites and their eggs.

### Collection of Fæces

Fæces are usually collected in a bed pan. For most examinations the complete stool should be sent to the laboratory within a short time after being passed. For some examinations such as tests for occult blood, it is more convenient to send a portion of the stool in a waxed carton. This should be a carefully selected part of the stool. Otherwise, and particularly for quantitative determinations, the whole stool is necessary. In most cases it is essential to avoid using stools which contain liquid paraffin, barium salts, or which have been collected after an enema has been given.

It is also in most cases important to ensure that admixture with

urine does not occur. Simple inspection as a rule will show whether or not this has occurred. A good indication is given by the presence of considerable amounts of chloride in contaminated stools (Cammidge). Chloride occurs only in small amounts in fæces, but may be present in high concentration in urine. A portion of the fæces is boiled with a small amount of distilled water, and the filtrate tested for chlorides with silver nitrate and nitric acid. Fæces give no more than a slight opalescence. A precipitate of varying degrees of denseness is produced if contamination with urine has occurred.

### Preservation of Fæces

For the most part specimens should be examined soon after being passed so that the need for preservation is avoided. If the fæces are to be dried, for example as a preliminary to fat determination, they should be transferred to a suitable dish and drying begun in an oven or on a water bath soon after the specimen is received. When metabolic experiments such as calcium balance tests are being done, it is also best to begin drying each specimen as received and then mix the powdered dried fæces intimately at the end, before taking a suitable amount for the estimation. In other cases it is possible to do the first stage of the examination at once, for example, in the estimation of urobilinogen, in which the appropriate quantity of fæces is mixed with the alkaline ferrous sulphate. The rest of the determination can be completed later.

If fæces have to be kept, they are best left in a refrigerator. When fæcal nitrogen is to be estimated, acid may be added. This is best done by adding about 200 ml. of water, mixing the fæces intimately so that a uniform suspension is obtained, taking a small, known volume of this and adding carefully with constant stirring an equal volume of concentrated sulphuric acid. This mixture can then be kept and a suitable amount taken for the analysis. Addition of a small amount of formalin has also been used for preserving fæces.

### Marking Fæces

In experiments such as nitrogen and calcium balances it may be necessary to collect all the urine and fæces produced over a period of time, usually for three or four days. Whilst collection of urine is straightforward, that of the fæces is more difficult. Several substances have been used to mark the fæces corresponding to the start and end of the experimental period. Charcoal, carmine and gentian violet have been most used. The dyes are the more successful, $\frac{1}{2}$ to 1 gram being a suitable quantity. This is given in capsules before breakfast on the first morning of the test, at the point when collection of urine is begun, and again before breakfast on the last day of the test, the point at which urine collection ceases. The first specimen of fæces to contain the dye is used, and all specimens up to but not including that which contains the second dose of dye.

### The Amount of Fæces

The quantity of fæces which is passed daily is very variable, being influenced by the type of diet and the amount of water taken. The

weight for a day's fæces ranges from 60 to 200 grams for adults. The bulk is increased if considerable amounts of vegetables are being eaten, when twice this amount may be excreted. Diets low in vegetables and rich in meats lead to a reduced quantity of fæces. A greatly increased bulk is found in some pathological conditions, particularly in steatorrhœa, when 500 to 1,000 grams may be passed daily. Such an increase is obvious on inspection.

### Water Content

On a typical mixed diet the fæces contain an average of 75 per cent. (70 to 80 per cent.) water. On a rich meat diet it tends to be lower, on a rich vegetable diet to be higher. The higher the fat content of the fæces, the higher the water content. In diarrhœa the water content is well over 90 per cent.

### Total Solids

The daily excretion of fæces expressed as the dried weight is consequently in the range 25 to 45 grams. It may be almost twice as great when considerable quantities of vegetables are being eaten. When fasting, the daily dried weight falls to about 2 to 3 grams.

### *p*H of the Fæces

The *p*H of the fæces varies from 5 to 9, again being considerably influenced by the diet. There are also considerable variations in a single stool. On an average the *p*H appears to be slightly on the alkaline side of neutral. Because of the wide variations found both in health and disease, determinations yield little or no information of value and are rarely made. Freshly passed specimens must be used. The simplest method is to use an indicator paper. British Drug Houses (Poole, Dorset) make several booklets of these covering suitable *p*H ranges, such as 4·0 to 5·5, 5·5 to 7·0, and 7·0 to 8·5. The paper is moistened with distilled water on a glass slide, and a small portion of the fæces rubbed on it.

### Odour

The odour of normal fæces is mainly due to the action of bacteria on proteins, such substances as indole and skatole being produced. The quantity formed is thus largely influenced by the amount of protein, particularly meat, in the diet. Other compounds containing sulphur, such as organic sulphur compounds and hydrogen sulphide, largely contribute to the odour of more disagreeably smelling fæces.

### Standard Diets for Collection of Fæces

Since the diet has such a great influence on the composition of the fæces, a standard diet is advisable if reliable comparative results are to be obtained. One such diet, widely used in the past is that of Schmidt (see, for example, Cammidge, 1914). This contains approximately 102 grams of protein, 111 grams of fat, 190 grams of carbohydrate, and has a caloric value of 2,230. The diet should be a bland one with a low residue. A number of rather similar diets have

been used. Thus Wollaeger, Comfort, and Osterberg (1947), used a diet containing 102 grams fat, 117 grams protein, and 270 grams of carbohydrate, with a calorie value of 2,532. Nothman (1951) gave a modified Schmidt diet which contained 105 grams of protein, 135 grams fat, 180 grams carbohydrate, calories 2,424.

## SOME NOTES ON COLORIMETRY

Owing to a number of factors such as the small amount of many of the substances which have to be determined in clinical biochemistry, and the difficulty of isolating them, colorimetric methods are frequently used. In the space available here it is not possible to give a full account of the theory of the subject and of the instruments used, but only to deal briefly with the more immediately relevant points. For more detailed accounts see Snell and Snell (1948) and Vogel (1951). For photoelectric colorimetry see Lothian (1949) Delory (1949), King (1942), and Müller (1939). In addition full descriptions of the instruments referred to below, with accounts of the method of using them, are usually available from the manufacturers. We shall consider in turn methods of direct visual comparison, visual colorimeters, and photoelectric instruments.

### Visual Comparison

One of the simplest methods, which can be used when no instrument is available, is to match the unknown against a set of standards, using test tubes of similar diameter. Values intermediate between successive standards can be assessed. The accuracy clearly depends on the number of standards used. If a series of standards has to be prepared each time, the method is more troublesome than the use of an instrument, but if a set of standards can be kept, the method is convenient and can give results which are sufficiently accurate for clinical purposes in many cases. This principle is used in the *Lovibond Comparator* in which the coloured solution is compared with permanent standard discs comprising a series of coloured glasses. These are available for most of the commoner methods used in the hospital laboratory. For a full list of these see for example Messrs. British Drug Houses' catalogue.

Another simple method is the *dilution method*. In this case a standard is prepared and the standard or the unknown diluted until they match in intensity when observed in similar test tubes by the naked eye. The concentration of the unknown can be easily calculated from the amount of dilution required. This is less accurate than the previous method since dilution may alter some of the conditions which influence the intensity of the colour, and this will only affect one of the colours which are being compared.

### Visual Colorimeters

Widely used for many years these have now been largely superseded by photoelectric instruments. The source of light is usually

in the base of the colorimeter so that light passes vertically in equal intensity through two cups, the sides of which are opaque and the base transparent. Each cup stands on a small platform which can be moved up and down. The cups contain the standard and unknown solutions respectively. Into each cup dips a cylindrical plunger transparent at the bottom and top but otherwise opaque. By moving the cups up and down it is thus possible to vary the depth of coloured solution through which the light passes. The plunger carriers are provided with graduated scales so that the depths of solution viewed can be read. The zero of these corresponds to the point at which the plunger just reaches to the bottom of the cup. After passing through the coloured liquids and the plungers, the two beams of light are brought by a prism into the eyepiece so that they can be observed together, each forming half of the circular area of colour seen there. A well-known example of this type of instrument is the Duboseq colorimeter. Messrs. Baird and Tatlock's microptic colorimeter is shown in Fig. 6.

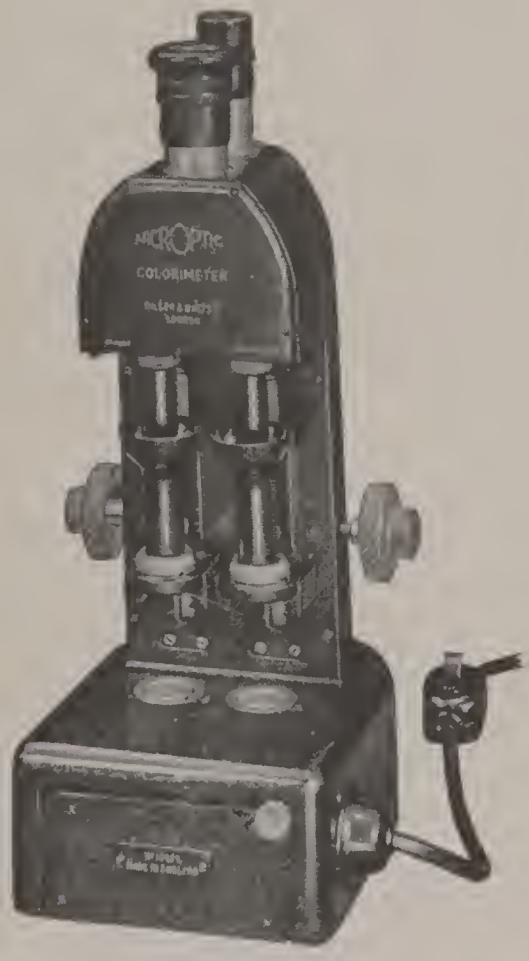


Fig. 6. The Microptic Colorimeter (Messrs. Baird and Tatlock).

The fundamental law on which visual colorimetry is based is Beer's law, which states that the intensity of colour of a solution when viewed by transmitted light depends on the number of coloured particles present, independently of their concentration. From this it follows that the same amount of colour is seen for example when a 10 per cent. solution is viewed through a depth of 4 cm. as when a 20 per cent. solution is viewed through 2 cm., that is *the intensity of the colour seen is proportional to the concentration of the substance* ($c$) *multiplied by the depth of the solution* ($t$). From this it follows that when two solutions of a substance viewed by transmitted light have the same intensity of colour

$$c_1 t_1 = c_2 t_2.$$

Hence if we introduce a suitable standard into one cup and the unknown into the other, adjust the platforms until the two match, and then read the depths of solution through which the light is passing,

Concentration of standard × Reading of standard
= Concentration of unknown × Reading of unknown

that is,
Concentration of unknown

$$= \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{Concentration of standard.}$$

The maximum depth of liquid which can be viewed in ordinary instruments is 40–50 mm. It is a common practice to set the unknown at 20 or 30 mm. and move the standard up and down until the two solutions match. This simplifies the calculation since a simple denominator is obtained.

In making the actual reading it is best to approach the point of matching from both sides, and to take several readings of which the average is calculated. It is not possible to eliminate the personal element completely.

A standard should be used which does not differ widely from the unknown. The ratio of the two colours should be less than 2 : 1 and the intensity of colour chosen for the matching should be one which is sensitive to small changes in depth of solution.

The instrument should be checked to see that the zero reading corresponds to the point at which the plunger just touches the bottom of the cup. The cups and plungers must be kept perfectly clean. It is a good practice to keep distilled water in the cups when they are not in use. When using, care should be taken not to put too much liquid in the cup or some may overflow. Enough liquid to fill the narrow part of the cup is sufficient. Finally it is necessary to make sure the plungers dip beneath the surface of the liquids when the readings are taken, and that a small air bubble is not trapped under the plunger.

## Photoelectric Colorimetry

The instruments used for this are not colorimeters but absorptiometers, since it is the amount of light absorbed which is measured as will be evident when the method of using them is discussed. The essential parts are :—

1. A source of light. The intensity of this differs considerably in the various instruments.

2. A means of selecting a sufficiently narrow wave band. In most instruments in use to-day filters are used for this purpose, but in some a diffraction grating is employed, and in others a prism. For really satisfactory work approximately monochromatic light is required.

3. Cells to hold the coloured solutions. Test tubes are used in some of the simpler instruments. They must be interchangeable. Whilst perhaps more convenient, they are not so satisfactory as the cuvettes with two parallel flat sides.

4. A photosensitive element. Most instruments use one or two *barrier layer cells*. In these a thin layer of selenium is deposited on to a metal disc, and is itself covered by a thin transparent layer of metal lacquered except for a thicker part near the periphery from

which a connection is made to the galvanometer. The other terminal of the galvanometer is connected to the back of the metal disc. The light passes through the thin metal layer to the layer of selenium beneath. Here it liberates electrons which pass across the "barrier" between the selenium and the thin metal layer, which thus becomes negatively charged and the thicker metal disc positively charged. If a galvanometer is connected across this cell a current passes, the strength of which is proportional to the intensity of the light falling on to the selenium. Among such cells is the EEL cell of Evans Electroselenium Ltd., which is sensitive to the visible spectrum. A range of cells sensitive to various regions of the spectrum is made by Megatron Ltd.

Other instruments use *photoemissive tubes*. These are similar in appearance to radio valves and are composed of a glass tube, either evacuated or containing an inert gas at low pressure, in which there are two elements, a cathode formed from a half cylinder metal plate coated on the inner surface with a light sensitive substance such as potassium or cæsium oxide, and a thin wire along the axis of the cylinder as anode for collecting the electrons emitted. Unlike the selenium cell which needs no external source of current, a potential has to be maintained at this anode for which purpose a battery supplying 30–100 volts is usually employed. The current from the phototube is smaller than that from the selenium cell but can be readily amplified, whereas in most cases that from the barrier layer cell cannot but is, however, usually large enough to be read satisfactorily. Phototubes can be obtained sensitive to wavelengths well beyond both ends of the visible spectrum, and they are free from some of the faults of the selenium cell.

5. A suitable means of measuring the output of the photosensitive element. In most instruments a sensitive galvanometer is used.

It will be seen that a variety of instruments is available. A convenient division is according to whether the instrument has one or two photosensitive elements. A further important distinction arises from the means used to obtain monochromatic light.

1. **Single Cell Instruments.** In these the current produced is measured directly on the galvanometer. A low level of illumination, usually 6 or 12 volts (but in the case of the EEL Portable Colorimeter, of only 2·2 volts), and a galvanometer of low resistance in the circuit external to the cell are necessary if there is to be a straight line relationship between the intensity of the light falling on to the cell and the amount of current produced. A constant voltage transformer must be used to remove fluctuations in the mains current employed as power for the source of light. Examples of this type of instrument are

(*a*) *Using Filters.* The EEL Portable Colorimeter and the EEL Absorptiometer made by Evans Electroselenium Ltd. ; the Biochem Absorptiometer of Hilger & Watts Ltd. ; and in the U.S.A. the Evelyn photoelectric colorimeter.

(*b*) *Using a Diffraction Grating.* The Unicam spectrophotometer

made by the Unicam Ltd., Cambridge, which provides a waveband 35 millimicrons wide ; in the U.S.A. the Coleman Junior spectrophotometer. In this type the part of the spectrum required can be selected by moving a dial calibrated in wave-lengths. For the visible spectrum this is 400 700 millimicrons.

2. **Double Cell Instruments.** In these, two closely matched selenium cells are balanced against each other so that no current passes through the galvanometer when the actual reading is being made. Fluctuations in the mains supply affect each side equally and so can be ignored. The best example in this country is the Spekker absorptiometer of Hilger & Watts Ltd. In the U.S.A. there is the Klett-Summerson made by the Klett Company. The circuits for these are outlined in Fig. 7. In the Klett Summerson (Fig. 7a) the potentials developed across the resistances in circuits A and B by the currents flowing through them are balanced against each other. The resistance in circuit A consists of a potentiometer with a calibrated dial. By varying this resistance it is possible to

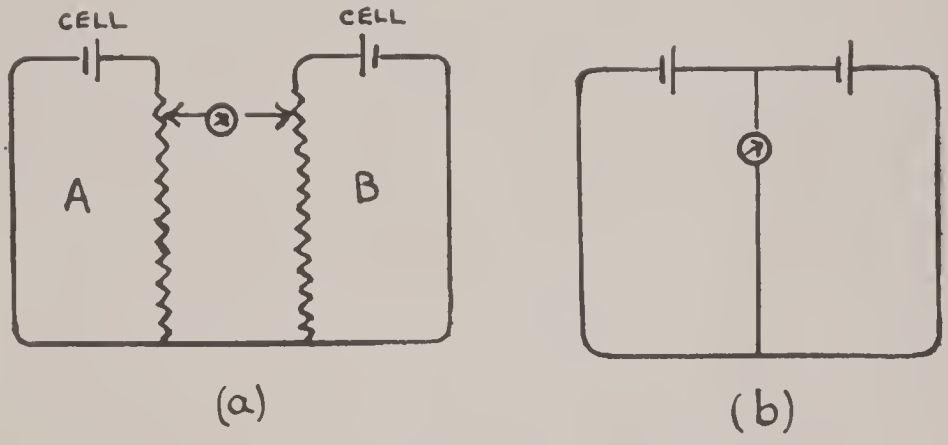


Fig. 7. Circuits of Double-Cell Absorptiometers.

counterbalance the effect of changes in the current flowing in circuit B arising from differences in the amount of light absorbed by the coloured solutions. The readings on the dial are usually in optical densities. In the Spekker (Fig. 7b) a calibrated drum (also in optical densities) placed in front of the cuvette into which the coloured solutions are placed, varies the amount of light which passes through. How these instruments are used will be referred to later.

In addition there are available spectrophotometers which read both in the visible spectrum and in the ultraviolet. Examples are the Unicam ultraviolet spectrophotometer, the Hilger Uvispek, and in the U.S.A. the Coleman universal and Beckmann spectrophotometers. Two light sources are necessary, one for the visible and red regions, and one for the ultraviolet. The Unicam uses a tungsten filament lamp and a hydrogen discharge lamp respectively. As monochromator a quartz prism is used. The range of wave-length covered is 200–1,010 millimierons. Two photocells are employed, a red sensitive for use from 1010–600 millimierons and one sensitive to the ultraviolet for the range 625–200 millimierons.

We shall now consider briefly the use of these instruments. When light passes through a coloured solution some of the light is absorbed. The two fundamental laws which express quantitatively the extent of this absorption are those of Lambert and Beer. From these is derived the equation

$$\frac{I_E}{I_0} = e^{-kct}$$

in which

$I_E$ = intensity of the emergent light,
$I_0$ = intensity of the incident light,
$k$ = a constant,
$c$ = concentration of coloured substance,
$t$ = thickness of the layer of solution,
$e$ = 2·718, the base of natural or Naperian logarithms.

This states that when monochromatic light passes through a coloured solution the amount of light transmitted decreases exponentially (*a*) with increase in thickness of the layer of solution through which the light passes, and (*b*) with increase in concentration of the coloured substance. The first of these follows from Lambert's law which states that the proportion of the light absorbed is independent of the intensity of light entering the solution. The same proportion of light entering it is thus absorbed by each successive layer. The second (*b*) follows from Beer's law stated earlier (p. 18).

The ratio $I_E/I_0$ is known as the *transmission* ($T$). Hence

$$T = e^{-kct}$$

or $$\log_e T = -kct$$

or $$-\log_e T = kct$$

or $$-\log_{10} T = Kct$$

Where $K$ is another constant.

The quantity —log T or log (1/T) is termed the *optical density* ($D$) or the *extinction* ($E$).

When using photoelectric instruments we substitute the solvent or a blank for the coloured test solution and measure the intensity of the emergent light from either of these. This can be denoted $I_B$ and is substituted for $I_0$ to give

$$\frac{I_E}{I_B} = e^{-kct}$$

$I_E/I_B$ has been termed the transmittance.

Some photoelectric instruments are provided with two scales, one from 0 to 100 showing percentage transmission (or transmittance), the

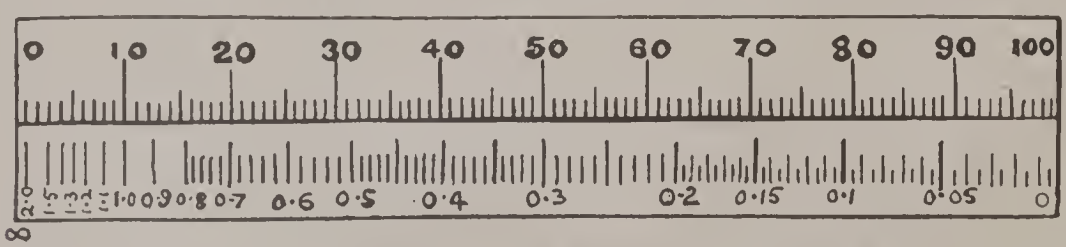


FIG. 8. Scales used on Photoelectric Instruments.

other from $\infty$ to 0 showing the optical density. These are shown in Fig. 8. It will be seen that while the first is linear, the latter is logarithmic and that the optical density and the per cent. transmission are related

$$D = \log \frac{100}{\text{per cent. transmission}}$$

or

$$D = 2 - \log \text{ per cent. transmission.}$$

In practice the instrument is usually set for 100 per cent. transmission or optical density 0 with water or a blank in position. The test solution is then introduced and the optical density again read. Since $D = -\log T$

$$D = Kct.$$

So if the thickness remains the same $D$ is proportional to the concentration. As a result, provided Beer's law is obeyed, when optical density is plotted against concentration a straight line is obtained. On the other hand, if per cent. transmission is read, as it is in some instruments, and is plotted against concentration, we do

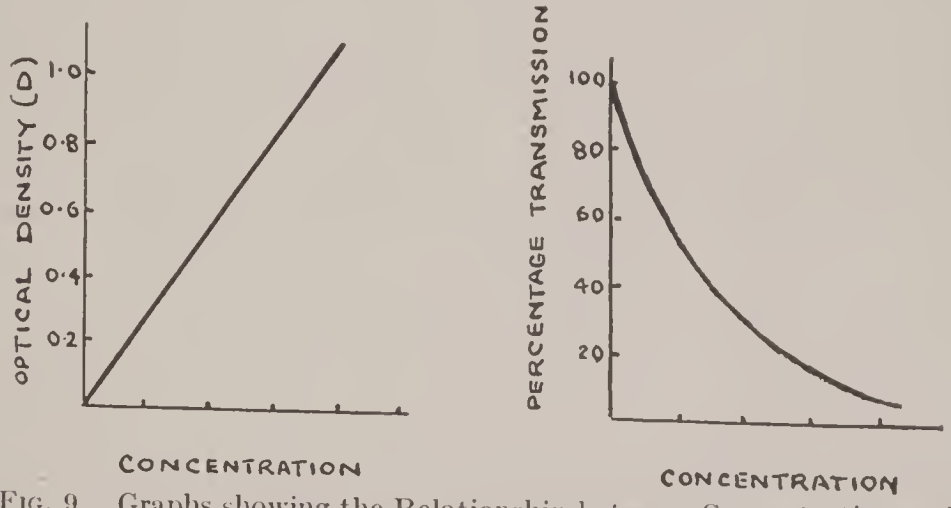


FIG. 9. Graphs showing the Relationship between Concentration and Optical Density, and between Concentration and Transmission.

not get a straight line but a curve as shown in Fig. 9. To convert this into a straight line semilogarithmic paper must be used with the concentration axis linear and the per cent. transmission logarithmic.

So we see that if a suitable standard is prepared and the optical densities of this and the unknown solution are read, we have

$$\frac{\text{Concentration of unknown}}{\text{Concentration of standard}} = \frac{\text{Optical density of unknown}}{\text{Optical density of standard}}.$$

So

$$\text{Concentration of unknown} = \frac{\text{Optical density of unknown}}{\text{Optical density of standard}} \times \text{Concentration of standard.}$$

Throughout this book this has been expressed :

$$\text{Concentration of unknown} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \text{Concentration of standard.}$$

This should be compared with the formula used with the visual colorimeter. It will be seen that the fraction is reversed for the latter. An example is given on pp. 31–2.

Since this formula only holds when Beer's law is obeyed, this should be verified by preparing a standard curve for the range of concentrations covered by the determination. Examples are given throughout the book. If this standard curve is not a straight line a standard close in colour to the unknown should be prepared.

It is emphasized that for accurate work a standard should be put up with each set of determinations. See Archibald (1950) and Henry and Segalove (1952). Many factors both in the reaction itself and in the behaviour of the photoelectric instrument operate to cause slight variations in the intensity of the colour produced at different times. These may sometimes add up to appreciable errors. Henry and Segalove discuss the question of recording daily or successive readings of the standard used so that the range of variation can be studied. From such charts we can see whether this is greater than is desirable if the required precision for the particular determination is to be achieved. Furthermore, trends in the reading of the standard may then be noted before they become serious. In certain circumstances, for example when a standard substance is difficult to obtain, it may be necessary to rely on a standard curve prepared previously. Evidence that the instrument is behaving consistently can be obtained if it is known that standards of approximately the same colour which are being used for other determinations are giving readings agreeing closely with those obtained on previous occasions.

In using photoelectric instruments satisfactory results are only obtained within a range of optical density from about 0·1 to 1·0 (better still between 0·2 and 0·7), so that the determination should be adapted to give a range the upper limit of which falls below the greater of these figures. Different instruments give readings of optical density which differ to a certain extent.

**Use of Blanks.** As already stated, in most cases a blank is prepared. With single cell instruments this is used to set for 100 per cent. transmission, that is optical density 0. The blank is then replaced in turn by the standard and the unknowns and the optical densities of these read. When reading a series of solutions it is advisable to check the 100 per cent. transmission setting at intervals. With two cell instruments a slightly different procedure is adopted. Thus in the Klett-Summerson type of instrument water is placed in both cuvettes and the galvanometer brought to zero thus setting the instrument for 100 per cent. transmission. The unknowns and standard are then read in turn against the blank. With the Spekker absorptiometer adjustments are made so that the galvanometer is at zero when the coloured solution is in position with the calibrated drum fully opened and thus reading 0. The other cell contains water. The

eoloured solution is then replaeed by the blank. More light passes. The galvanometer is brought baek to the zero by moving the ealibrated drum so as to admit less light. The reading on the drum then eorresponds to the amount of light previously absorbed by the eoloured solution. A series of solutions ean be read in turn if the most intensely coloured is put in first. Each reading has then to be subtraeted from the reading of the blank.

In some eases it is neeessary to put up two blanks, one to read the standard against, the other a full reagent blank to read the unknowns against. Alternatively the standard, the unknowns, and the reagent blank ean be read against the standard blank and the reading of the reagent blank subtraeted from the reading of the unknown. An example is provided by the urease-nesslerisation method for determining blood urea given on p. 105. In this a standard ammonium sulphate solution is treated with Nessler's reagent, the blank being prepared by treating ammonia-free water in the same way. The reagent blank is prepared by earrying out the full test, but substituting water for the blood. If standards are prepared by earrying out the full determination, for example in this ease on solutions of known urea eontent, only a reagent blank need be prepared. In some determinations the standard blank and the reagent blank are identieal. In the urease-nesslerisation method this is so if the reagents used are all ammonia-free. Then only a standard blank need be prepared. In other determinations the standard blank may give the same reading as water so that the setting ean be made with water. These points should be borne in mind in all the eolorimetrie methods described.

**Filters.** Ilford speetrum filters (603–609) and bright speetrum filters (621–626) are widely used. These are of gelatin eontaining suitable dyes. Curves whieh show the pereentage transmission at different wave-lengths for these filters are given in Fig. 10. It will be seen that maximum transmission of the speetrum filters is as follows :

| | | | | |
|---|---|---|---|---|
| 600 | Spectrum | deep violet | 420 | millimicrons |
| 601 | ,, | violet | 430 | ,, |
| 602 | ,, | blue | 470 | ,, |
| 603 | ,, | blue green | 490 | ,, |
| 604 | ,, | green | 520 | ,, |
| 605 | ,, | yellow green | 550 | ,, |
| 606 | ,, | yellow | 580 | ,, |
| 607 | ,, | orange | 600 | ,, |
| 608 | ,, | red | 680 | ,, |
| 609 | ,, | deep red | 700 | ,, |

The eurves also show that these filters have narrow transmission bands and so transmit approximately monochromatie light.

Sometimes it is necessary in order to get full scale deflection of the galvanometer to use filters with a broader wave band so that they transmit more light. Examples among Ilford filters are : Minus blue, 110 ; minus red, 302 ; minus green, 503 ; tricolour red, 204 ;

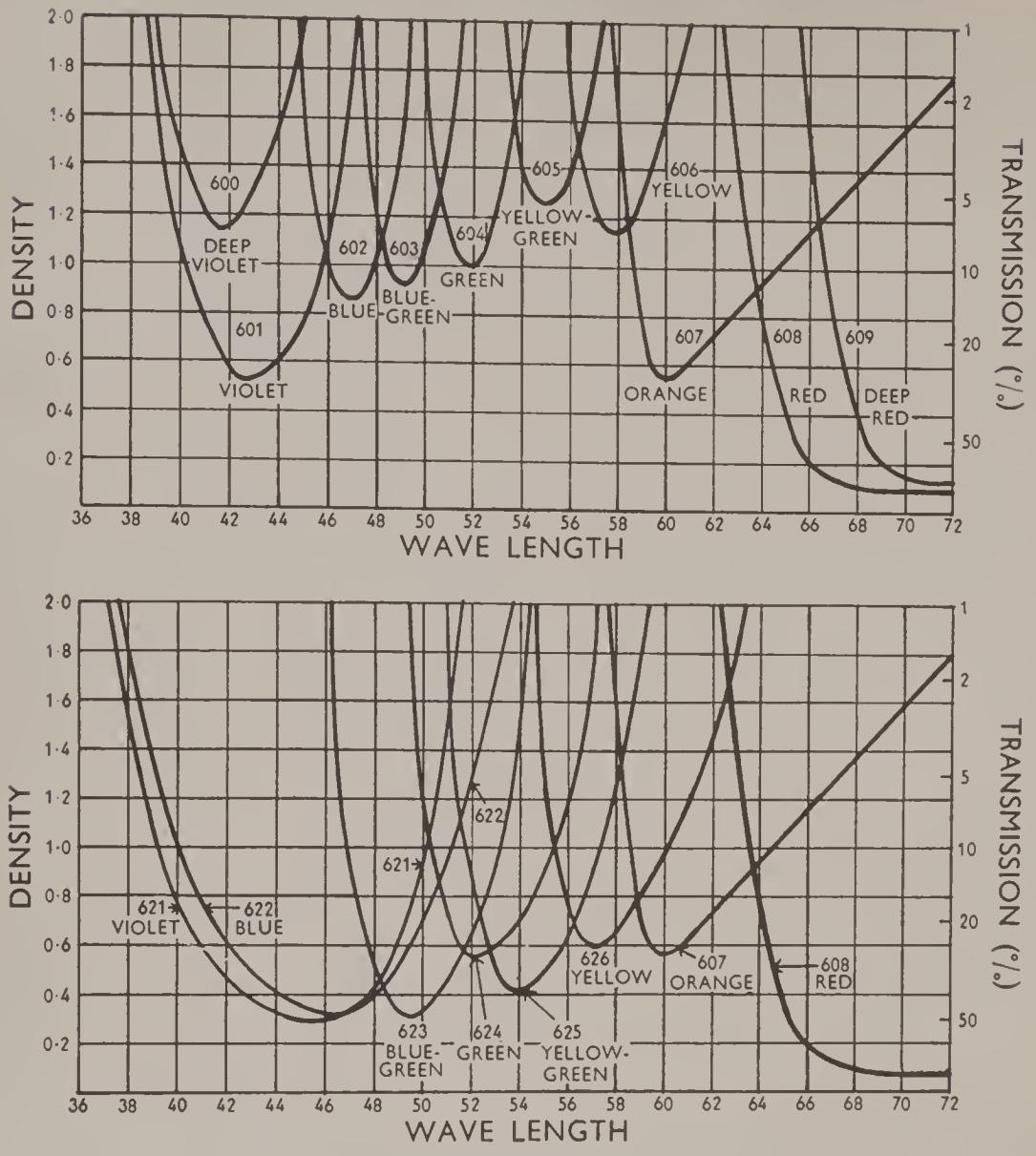


FIG. 10. Transmission Curves of Ilford Spectrum Filters and Bright Spectrum Filters.

(Abscissæ × 10 = millimicrons.)

tricolour blue, 304 ; tricolour green, 404. This latter and narrow cut tricolour red, 205. and a blue filter micro 2, No. 303, are supplied with the EEL Portable Colorimeter. The per cent. transmission curves for these filters are shown in Fig. 11. Tables giving the per cent. transmission of Ilford filters for all wave-lengths from 350–720 millimicrons are given in the booklet "Ilford Filters," published by Ilford Ltd.

Other filters used have included the Chance All Glass filters.

In selecting an appropriate filter two conditions should, if possible, be fulfilled. The wave-length chosen should be such that there is a straight-line relationship between optical density and concentration, and secondly the rate of change of optical density with concentration, that is the gradient of the line, should be great enough to give the required sensitivity.

Although there are occasional exceptions, in general the filter used is that which allows maximum absorption for the colour produced in

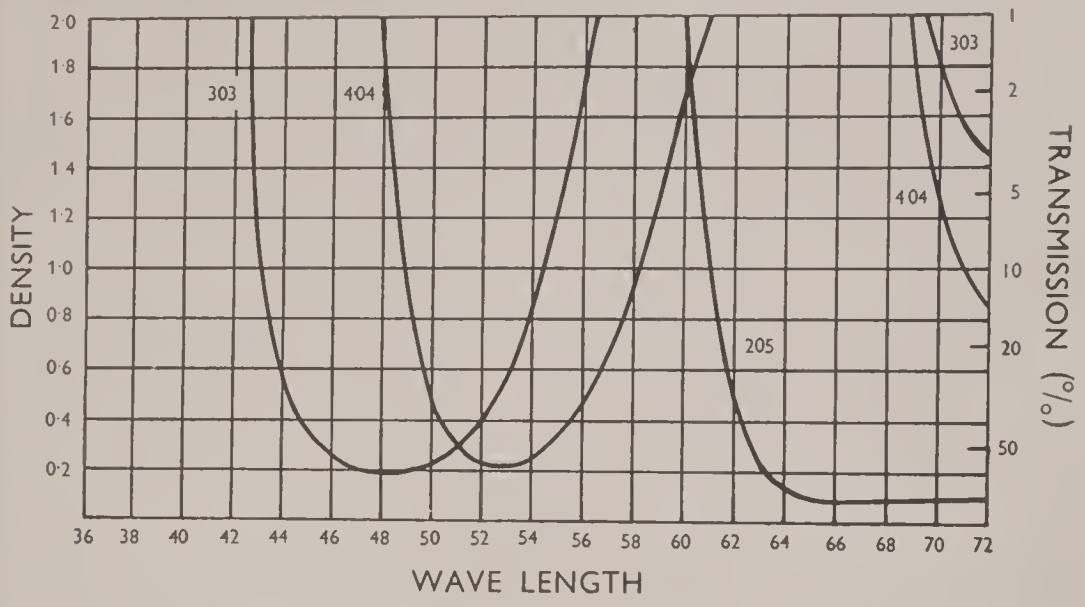


FIG. 11. Transmission Curves of Ilford Filters used with the EEL Portable Colorimeter.
(Abscissæ × 10 = millimicrons.)

the determination. This is usually the complementary colour. Thus for red to orange we use blue to blue green filters, for green a red filter, for blue a red filter, for purple a green filter, for yellow a violet filter. If the absorption is measured with each of the range of filters in position, we obtain an absorption curve, an example of which is shown

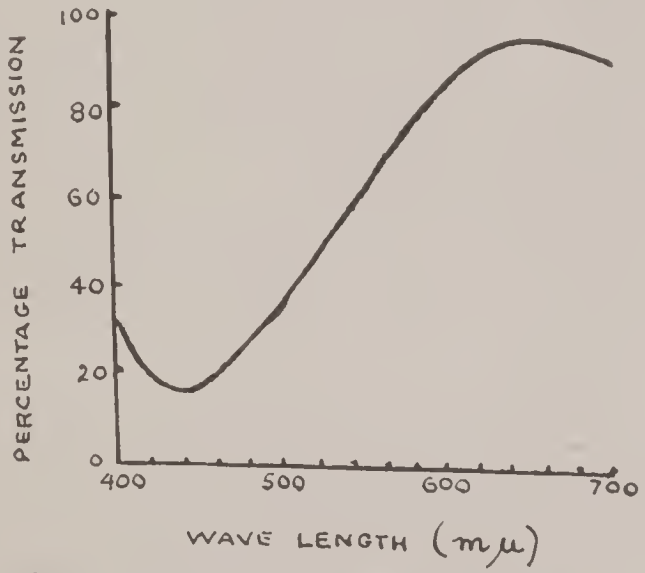


FIG. 12. Example of Absorption Curve.

in Fig. 12. From this the filter which gives the maximum absorption can be found. To test the suitability of such a filter a standard curve can be put up covering the range of concentration required. A simpler method is to put up only two solutions, one corresponding

to the maximum concentration it is desired to measure, the other half this concentration. The optical density of the second should be half that of the first.

If an instrument with a diffraction grating is being used the procedure is similar. An absorption curve can be prepared by reading the optical density at intervals of 20 millimicrons over the range covered by the instrument (ordinarily 400–700 millimicrons). The wave-length of maximum absorption is thus obtained and its suitability checked as described above for the filter chosen in the same way.

**Note on the Volume of Liquid Used.** The depth of liquid ordinarily used in these instruments is 1 cm. The volume of liquid required to give this differs appreciably in the various instruments mentioned, but is usually between 3 and 8 ml. In most cases the methods described later can be easily adapted to give the required volume.

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*CHAPTER II*

# BLOOD SUGAR AND ITS DETERMINATION

Since the introduction of insulin for the treatment of diabetes mellitus, the determination of blood sugar has been one of the tests most frequently carried out in the biochemical laboratory. The great majority of methods are done in three stages:

1. Precipitation of the blood proteins.
2. Reduction by the glucose, either of an alkaline copper sulphate solution to cuprous oxide, or of alkaline potassium ferricyanide to ferrocyanide.
3. Estimation of the amount of such reduction and thus of the amount of glucose present, either iodometrically or colorimetrically.

## Non-glucose Reducing Substances

These are present in blood up to an amount equivalent to 20 to 30 mg. glucose per 100 ml. of blood. Glutathione is the most important of these substances, which are present mainly in the cells. The different blood sugar methods include varying amounts. The method of precipitating the proteins, and the nature of the alkaline copper or ferricyanide reagent used, are important in determining the amount included in the glucose estimation. Determination of true glucose does not include any. It is claimed that such a value is given if the blood is placed directly into an isotonic sodium sulphate-copper sulphate solution in which the cells are not hæmolysed. Diffusible substances pass into the solution whereas on adding the protein precipitant the cell envelopes, which contain most of the non-glucose reducing substances, are carried down with the precipitate (Folin, 1930; Herbert and Bourne, 1930 ; King and Pillai and Beale, 1941). Benedict (1931, *a*), however, argues in favour of using whole blood. He has produced a reagent almost unaffected by these non-glucose reducing substances and claims that his method estimates true glucose in whole blood (Benedict, 1931, b ; see also Sunderman and Fuller, 1951).

## Glycolysis

Glucose disappears fairly rapidly from whole blood on standing, so that half the glucose may be lost in two to three hours. This is due to its conversion into lactic acid $C_6H_{12}O_6 \longrightarrow 2\ C_3H_6O_3$ a process, known as glycolysis, which occurs even in sterile blood. This can be prevented by adding sodium fluoride to the anticoagulant. A mixture of sodium fluoride and potassium oxalate in the proportion of one part to three parts will prevent any loss of glucose for two to three days, so that such blood can be sent through the post. An excess should not be used, since it may interfere with the estimation. Twenty milligrams of the mixture per 5 ml. of blood is adequate.

When capillary blood is used this is placed immediately into a solution which precipitates the protein. When this has been done there is no further possibility of glycolysis occurring. Blood placed into the isotonic sodium sulphate-copper sulphate solution referred to above and described p. 33, will also maintain its glucose content for at least forty-eight hours.

### Determination of Blood Sugar

Since the estimation of blood sugar is so frequently carried out and there are a number of methods in use satisfactory for ordinary routine work, several of these will be described.

The literature on the estimation of blood sugar is very considerable. Among the leading workers are Folin, Benedict and Somogyi in America, whilst in England, King has made several contributions to the subject. A number of references will be given, and it is to these that the reader is referred if he desires to learn more of the considerations which have led to the numerous modifications in reagents and techniques which have been suggested.

We shall consider first some of the colorimetric methods which have been used. Although both copper and ferricyanide methods have been adapted for this purpose, the former have been more frequently used. The copper method originally described by Folin, either in its first form or in subsequent modifications, has been and continues to be, the most widely used of all blood sugar methods. The original technique is still used in many laboratories and so will be given first and then will be followed by some of the later modifications and adaptations.

#### I. Method of Folin and Wu (Folin and Wu, 1920)

Slightly modified and adapted for the use of capillary blood.

Proteins are removed by the standard Folin-Wu tungstic acid precipitation. This is followed by reduction of an alkaline copper solution, the amount of cuprous copper formed being estimated colorimetrically by means of the blue colour obtained on addition of Folin's phosphomolybdic acid reagent.

**Reagents.** 1. Sodium tungstate, 10 per cent. solution. Dissolve 10 grams of $Na_2WO_4$, $2H_2O$ in water and make up to 100 ml.

2. Sulphuric acid, 2/3 N. (see p. 8).

3. Alkaline copper solution. Dissolve 40 grams of sodium carbonate (anhydrous) in about 400 ml. of water in a litre volumetric flask. Add 7·5 grams of tartaric acid. When this has dissolved, add slowly, with shaking, 4·5 grams of copper sulphate ($CuSO_4$, $5H_2O$) previously dissolved in about 100 ml. water. Mix, make to the mark with water, and mix again. If a sediment forms on keeping, remove by filtering or decant the supernatant fluid.

4. Phosphomolybdic acid reagent. Dissolve 35 grams of molybdic acid and 5 grams of sodium tungstate in 200 ml. of 10 per cent. sodium hydroxide and 200 ml. of water and boil to remove ammonia present in the molybdic acid. This usually takes up to thirty to forty minutes. Cool, transfer with washing to a 500-ml. flask, thus diluting

to about 350 ml. Then add 125 ml. of phosphoric acid (S.G. 1·75) and make to 500 ml. with water.

5. Stock standard glucose solution, 0·1 per cent. Dissolve 100 mg. of pure glucose in saturated benzoic acid solution (0·25 per cent.) and make up to 100 ml. with the benzoic acid.

6. Standard glucose solutions for use. Prepare :

(*a*) A solution containing 0·025 mg. per ml. by diluting 2·5 ml. of stock standard to 100 ml. with the benzoic acid.

(*b*) A solution containing 0·05 mg. per ml. by diluting 5·0 ml. of stock standard to 100 ml. with the benzoic acid.

**Technique.** Take 0·1 ml. of capillary blood and place into 3·5 ml. of water, washing out the blood by sucking the water in and out of the pipette several times. Add immediately 0·2 ml. of 10 per cent. sodium tungstate and 0·2 ml. 2/3 N sulphuric acid, stand a few minutes, and filter or centrifuge. Pipette 2 ml. of the filtrate ( = 0·05 ml. of blood) and 2 ml. of the appropriate standard into Folin tubes (Fig. 13). To each add 2 ml. of the alkaline copper solution and place in a bath of boiling water for eight minutes. Then transfer to a beaker of cold water for one to two minutes. Add 2 ml. of phosphomolybdic acid reagent. Mix, and after standing for two minutes dilute to the 12·5 ml. mark with water. Compare the colours in the colorimeter. For the photoelectric colorimeter put up a blank from 2 ml. of water and 2 ml. of alkaline copper solution, carrying this through as for the test. Set the colorimeter with this when using one-celled instruments, read the unknown against it with two-celled ones. Use a red filter or transmission at 680 millimicrons.

Fig. 13. Folin Sugar Tube.

**Calculation.** (*a*) *Photoelectric Colorimeter.* For use with each batch of determinations, standard (*b*) is suitable. This contains 0·1 mg. glucose per 2 ml. Hence :

Mg. glucose per 100 ml. blood

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}1 \times \frac{100}{0{\cdot}05}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 200.$$

A standard curve can be prepared, using a standard solution containing 0·1 mg. per ml. obtained by diluting the stock standard 10 ml. to 100 ml.

| Mg. glucose per 100 ml. blood | 0 | 50 | 100 | 150 | 200 | 250 | 300 |
|---|---|---|---|---|---|---|---|
| Ml. standard solution (1 ml. = 0·1 mg.) | 0 | 0·25 | 0·5 | 0·75 | 1·0 | 1·25 | 1·5 |
| Ml. benzoic acid solution (or water) | 2·0 | 1·75 | 1·5 | 1·25 | 1·0 | 0·75 | 0·5 |

Proceed as for the test, with the colour development.

For results over about 300 and up to 600 mg. per 100 ml. dilute the final colour to the 25 ml. mark, and make the necessary adjustment in the calculation. For higher values use a smaller volume of filtrate, 1 ml. or 0·5 ml. and make up to 2 ml. with water before adding the alkaline copper solution.

(*b*) For the *visual colorimeter*. In this case it is best to set up both standards (*a*) and (*b*) given above. Then :

Mg. glucose per 100 ml. blood

$$= \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 100 \text{ for standard } (a).$$

$$= \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 200 \text{ for standard } (b).$$

For results from 300 to 600 mg. per 100 ml. dilute the colour to the 25 ml. mark and make the necessary allowance in the calculation. For higher values use less filtrate.

NOTES

1. *Use of the Ordinary Folin-Wu Filtrate.* Since this is a 1 in 10 dilution of blood instead of the 1 in 20 used above, take 1 ml. with 1 ml. of water, add 2 ml. alkaline copper solution and proceed as described above.

2. The special Folin tube is designed to minimize re-oxidation of the cuprous oxide formed. The surface of the liquid during the reduction should be in the narrow part of the tube, thus exposing only a small surface to the air.

3. The tartrate is used to hold the copper in solution, but the greater the amount of tartrate present, the greater the tendency for self-reduction to occur. It is to prevent this that in later modifications the reagent was prepared in two separate solutions to be mixed freshly for use.

4. *Modifications* (Folin, 1926, 1929). (*a*) *The alkaline copper solution.* Prepare two solutions :

> Solution A. Place 35 grams of anhydrous sodium carbonate in a litre volumetric flask, add 200 ml. of water and shake to dissolve. Add 13 grams of sodium tartrate and 11 grams of sodium bicarbonate and water to 800 ml. When dissolved, make up to the mark.
>
> Solution B. Five per cent. copper sulphate ($CuSO_4$, $5H_2O$) containing a drop of concentrated sulphuric acid.

Prepare freshly for use by adding 5 ml. of B to about 25 ml. of A in a 50-ml. volumetric flask or stoppered cylinder and make to the mark with solution A. This will keep for a few days in the ice chest.

(*b*) *The Phosphomolybdic Acid Reagent.* For rapid preparation of a modified reagent dissolve 40 grams of sodium molybdate in about 100 ml. of water in a 500-ml. beaker. Some turbidity may remain. Add, with stirring, 55 ml. of 85 per cent. phosphoric acid, 40 ml. of 25 per cent. (v/v) sulphuric acid and 20 ml. of 99 per cent. acetic acid.

The solution is then ready for use. Depending on the purity of the sodium molybdate, this solution tends to turn blue any time after a few days, and so may need preparing at frequent intervals. To prepare a purified reagent, dissolve 150 grams of sodium molybdate in 300 ml. of water. Filter into a litre flask, washing in with about 75 ml. of water. Add 3 to 4 drops of bromine and shake to dissolve. Stand an hour, then add, with shaking, 225 ml. of 85 per cent. phosphoric acid, followed by 150 ml. of cooled 25 per cent. v/v sulphuric acid. Remove the surplus bromine by means of a moderately rapid air current passed for about half an hour. Finally, add 75 ml. of 99 per cent. acetic acid, mix, and dilute to 1 litre.

The use of the original Folin phosphomolybdic acid reagent has however continued, both in the Folin-Wu method, and in other methods, for example, by Haslewood and Strookman, who precipitate proteins by placing the blood into the isotonic sodium sulphate copper sulphate solution used by King *et al* (1937).

5. Trouble has been experienced from fading of the colour. Several methods of overcoming this have been suggested. Thus Tonks (1952) does not cool after carrying out the copper reduction, but adds the phosphomolybdic acid immediately to the hot mixture, and then replaces the tube in the boiling water bath for a further two minutes before cooling finally.

### II. Method of Haslewood and Strookman (1939)

**Reagents.** 1. Isotonic sodium sulphate-copper sulphate solution. Prepare two solutions :

A. Copper sulphate, 7 per cent. solution. Dissolve 7 grams of $CuSO_4$, $5H_2O$ in water and make up to 100 ml.

B. Sodium sulphate, 3 per cent. solution. Dissolve 30 grams of $Na_2SO_4$, $10H_2O$ in water and make up to a litre.

To obtain the reagent as used, mix 30 ml. of A. and 320 ml. of B.

2. Sodium tungstate, 10 per cent. solution. Dissolve 10 grams of $Na_2WO_4$, $2H_2O$ in water and make up to 100 ml.

3. Alkaline copper solution. This is the Schaffer-Hartmann reagent as modified by Harding and Downs (1933) and used with addition of potassium iodate by King in his titrimetric method. Prepare freshly at frequent intervals by mixing equal quantities of the following two solutions :

A. Copper sulphate solution. Dissolve 13 grams of $CuSO_4$, $5H_2O$ in water and make up to a litre.

B. This contains 24 grams sodium potassium tartrate (Rochelle salt), 40 grams sodium carbonate (anhydrous), 50 grams sodium bicarbonate, and 36·8 grams of potassium oxalate. Dissolve the bicarbonate in about 600 ml. of water in a litre flask at room temperature. Wash in the carbonate and shake to dissolve, again without heating. Weigh out the oxalate, dissolve in several small amounts of warm water, pouring each time into the carbonate bicarbonate mixture, until it has all been added. Dissolve the sodium potassium tartrate separately in a small

amount of water in a beaker and wash in. Finally make up to the mark and mix well.

4. Phosphomolybdic acid reagent, prepared as in the original Folin method, p. 30.

5. Stock standard glucose solution, containing 100 mg. in 100 ml. saturated benzoic acid solution.

6. Standard solutions for use, prepared as indicated below.

**Technique.** Place 0·1 ml. of blood into 3·7 ml. of the isotonic sodium sulphate-copper sulphate solution. Add 0·2 ml. of 10 per cent. sodium tungstate, mix well, and centrifuge. Half these quantities can be used if desired. Pipette 1 ml. of the supernatant fluid (= 0·025 ml. blood), and add to it 1 ml. of the alkaline copper solution in a 6 × $\frac{3}{4}$ inch test tube. Plug the tube lightly with cotton wool and heat for ten minutes in boiling water. Cool immediately, and add 3 ml. of phosphomolybdic acid reagent. Make up to 10 ml. with water. Compare in the colorimeter with suitable standards.

For the photoelectric colorimeter, use as standard a solution prepared by diluting 5 ml. of the stock standard to 100 ml. This contains 0·05 mg. glucose per ml. Take 1 ml., add 1 ml. of alkaline copper solution and proceed as for the test. Then :
Mg. glucose per 100 ml. blood

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}05 \times \frac{100}{0{\cdot}025}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 200.$$

Put up a blank using 1 ml. water and 1 ml. alkaline copper solution treated in the same way as the test.

A standard curve can be prepared as follows :

| | | | | | |
|---|---|---|---|---|---|
| Mg. glucose per 100 ml. blood . . . | 0 | 100 | 200 | 300 | 400 |
| Ml. stock standard diluted to 100 ml. . | 0 | 2·5 | 5·0 | 7·5 | 10·0 |

Take 1 ml. of these, add 1 ml. alkaline copper and proceed as for the test.

This technique is suitable for values of blood sugar up to about 300 mg. per 100 ml. For higher values use less supernatant fluid, e.g., 0·5 ml., or further dilution.

**Nelson's Arsenomolybdate Reagent.** Nelson (1944) devised an arsenomolybdate reagent for use instead of the phosphomolybdate reagent given above. This has been used in several blood sugar methods. Thus Nelson used it with a Somogyi copper reagent (given later on p. 454), whilst King and Garner (1947) substituted this for the Folin phosphomolybdic acid reagent in Haslewood and Strookman's method, using 1 ml. instead of 3 ml. of the latter.

*Nelson's Arsenomolybdate Reagent.* Dissolve 25 grams of ammonium molybdate in about 450 ml. of water, add 21 ml. of concentrated sulphuric acid, mix, add 3 grams of disodium hydrogen arsenate

($Na_2HAsO_4.7H_2O$) dissolved in 25 ml. of water. Mix, and place in an incubator at 37° C. for twenty-four to forty-eight hours. If a reagent is needed quickly, the mixture may be heated to 55° C., but should be well stirred to prevent local overheating. It is better to incubate. Keep the reagent in a glass-stoppered bottle.

### III. Benedict's Method (Benedict, 1931, b)

Benedict devised a copper reagent to which he added sodium bisulphite. Non-glucose reducing substances have practically no effect on this. Benedict used a tungstomolybdic acid solution for precipitating the proteins, but the Folin-Wu filtrate can be used.

**Reagents.** 1. Tungstomolybdate solution. Dissolve 10 grams of pure ammonia-free molybdic acid in 50 ml. of normal sodium hydroxide. Boil gently for five minutes. Filter, washing the filter paper with 150 ml. of hot water. Cool the whole of this filtrate and add to it 80 grams of sodium tungstate dissolved in 600 ml. of water. Mix and dilute to 1 litre.

2. Sulphuric acid, 0·62 N. Prepare from stock normal solution.

3. Tungstomolybdic acid solution. Pipette 5 ml. of the tungstomolybdate solution into a 250 ml. volumetric flask, add 150 ml. of water and 5 ml. of 0·62 N sulphuric acid, mix, and make to the mark. Prepare freshly every three to five days.

4. Copper reagent. Dissolve 15 grams of anhydrous sodium carbonate, 3 grams of alanine, and 2 grams of Rochelle salt in about 350 ml. of water and add with constant stirring 3 grams of copper sulphate ($CuSO_4.5H_2O$) dissolved in 80 ml. of water. Dilute to 500 ml. This will keep in the refrigerator for one to two months.

5. One per cent. sodium bisulphite solution.

6. Copper-bisulphite reagent. Mix 10 ml. of the copper reagent and 1 ml. of the bisulphite solution. Prepare freshly each day.

7. Phosphomolybdic acid reagent. Add, with shaking, 500 ml. of water to 150 grams of pure ammonia-free molybdic acid and 75 grams of anhydrous sodium carbonate and dissolve, using heat if necessary. Filter from any insoluble material, washing the filter paper with about 100 ml. of water. Add 300 ml. of 85 per cent. phosphoric acid to the combined cooled filtrate, mix, and make to a litre.

8. Standard glucose solution. Prepare a stock solution containing 100 mg. in 100 ml. of saturated benzoic acid (see p. 31) and dilute this 1 in 20 to obtain the standard for use. This contains 0·05 mg. per ml.

**Technique.** Measure 4·9 ml. of tungstomolybdic acid solution (3) into a centrifuge tube and add 0·1 ml. of blood. Mix well, stand for at least a minute, and centrifuge. Transfer 2 ml. of the supernatant fluid into a Folin-Wu tube, add 1 ml. of the copper-bisulphite reagent and 1 ml. of water. Treat 2 ml. of the weak standard similarly. Heat in vigorously boiling water for five minutes, cool, and add 2 ml. of the phosphomolybdic acid reagent. Dilute to the 12·5 ml. mark, mix and read in the colorimeter, using either an orange or red filter.

**Calculation.** Since 2 ml. of the protein-free supernatant fluid is

equivalent to 0·04 ml. of blood, and 2 ml. of the weak standard contains 0·1 mg. glucose, for the photoelectric colorimeter,

Mg. glucose per 100 ml. blood

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}1 \times \frac{100}{0{\cdot}04}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 250$$

For values from approximately 300 to 600 mg. per 100 ml. make up to the 25 ml. mark.

**Notes.** 1. Sunderman and Fuller (1951) have modified Benedict's method. They use a specially designed tube, and prevent fading by adding the phosphomolybdic acid to the hot mixture after carrying out the reduction, and then replace in the boiling water for a further three minutes before finally cooling and reading in the colorimeter.

2. The Folin-Wu tungstic acid filtrate can be used. Add 0·1 ml. of blood to 3·5 ml. of water and then add 0·2 ml. of 10 per cent. sodium tungstate and 0·2 ml. of 2/3 N sulphuric acid. Stand, centrifuge, and take 2 ml. of the supernatant fluid. Since this corresponds to 0·5 ml. of blood the calculation is

Mg. glucose per 100 ml. blood

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 200.$$

### IV. Method of Somogyi (1945, 1952)

Somogyi has suggested a modified alkaline copper tartrate reagent which it is claimed has a high degree of stability on keeping, whilst the high content of sodium sulphate and the absence of iodide prevent reoxidation of the cuprous oxide formed. Somogyi also substitutes barium hydroxide for sodium hydroxide for use with zinc sulphate in precipitating proteins.

**Reagents.** 1. Zinc sulphate. 5 per cent. solution of $ZnSO_4$, $7H_2O$.

2. Barium hydroxide, 0·3 N. Add 15 grams of barium hydroxide (or 28 gm. of $Ba(OH)_2 . 8H_2O$) to 500 ml. of hot water and boil for a few minutes. Stopper and allow to cool. Filter.

These two solutions should exactly neutralize each other with phenolphthalein as indicator. Place 10 ml. of the zinc sulphate in a flask, dilute to about 100 ml. with water, and titrate slowly with the barium hydroxide, shaking well, until a pink colour is obtained lasting for at least a minute. Adjust the hydroxide.

3. Alkaline copper tartrate reagent. Dissolve 24 grams of anhydrous sodium carbonate and 12 grams of Rochelle salt in 250 ml. of water. Add, with stirring, 4 grams of copper sulphate ($CuSO_4$, $5H_2O$) dissolved in about 50 ml. of water, followed by 16 grams of sodium bicarbonate. Dissolve 180 grams of anhydrous sodium sulphate in about 500 ml. of water, boil to expel air, cool, mix with the other solution, and make to a litre. Stand for a few days. Filter if necessary. Keep in a warm room.

4. Nelson's arsenomolybdate reagent. As above, p. 34.

5. Standard glucose solutions. The weak standards (*a*) and (*b*) used in the Folin-Wu method given on p. 31 are suitable.

**Technique.** Place 0·1 ml. of blood in 3·5 ml. of water and add 0·2 ml. of 0·3 N barium hydroxide and 0·2 ml. of 5 per cent. zinc sulphate. Shake well and filter or centrifuge. Pipette 1 ml. of the filtrate (= 0·025 ml. blood) into a 6 × $\frac{3}{4}$ inch test tube, add 1 ml. of water and 2 ml. of the alkaline copper solution and heat in a boiling water bath for ten minutes. Cool, add 1 ml. of the arsenomolybdate reagent and make up to 10 ml. with water. Compare in the colorimeter with suitable standards similarly treated. One ml. of the Folin-Wu (b) standard corresponds to 200 mg. glucose per 100 ml. of blood if treated in the same way as the test. Prepare a standard curve for the photoelectric colorimeter, using volumes from 0 to 2·0 ml. of this standard. This covers the range from 0 to 400 mg. glucose per 100 ml. blood. For values over about 300, however, it is usually better to use less supernatant fluid or dilute the final colour further.

**Notes.** For use as an *iodometric method* prepare the copper reagent as follows. Dissolve 30 grams of Rochelle salt and 30 grams of anhydrous sodium carbonate in 200 ml. of hot water. Add 40 ml. of N sodium hydroxide and then, with stirring, 8 grams of copper sulphate dissolved in about 80 ml. of water. Boil to expel air. Then pour in 180 grams of anhydrous sodium sulphate dissolved as above in 500 ml. of water. To the mixture add 8 grams of potassium iodide and 0·50 gram of potassium iodate, each dissolved in a little water. Make to a litre and mix.

For the test heat 2 ml. of 1 in 10 blood filtrate with 2 ml. of copper reagent in boiling water for ten minutes. Cool, add 1 ml. of 2 N sulphuric acid. Titrate with N/400 thiosulphate. As blank titrate 2 ml. of copper reagent plus 2 ml. of acid. The difference between the titrations gives a measure of the glucose present. Prepare a standard curve as shown in Table I.

TABLE I

*Standards for Somogyi Method*

| Mg. glucose per 100 ml. blood | 0 | 20 | 40 | 60 | 80 | 120 | 160 | 200 | 250 | 300 | 350 | 400 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ml. glucose solution (40 mg. per 100 ml.) | 0 | 0·1 | 0·2 | 0·3 | 0·4 | 0·6 | 0·8 | 1·0 | 1·25 | 1·5 | 1·75 | 2·0 |
| Ml. water | 2·0 | 1·9 | 1·8 | 1·7 | 1·6 | 1·4 | 1·2 | 1·0 | 0·75 | 0·5 | 0·25 | 0 |

Add 2 ml. of copper reagent to each and carry through as for the test. The range of titrations is from about 11·2–1·6 ml.

Variations can easily be made. If a 1 in 20 blood filtrate is used, the above standards range 0–800. This range can also be covered using a 1 in 10 filtrate if twice as much iodate is included in the copper reagent and N/200 thiosulphate for the titration.

## V. The Modified Schaffer-Hartman Method of King

(King *et al.* 1937, 1942.) Slightly modified

The blood is added to an isotonic sodium sulphate-copper sulphate solution from which the proteins are precipitated by the addition of

sodium tungstate. After centrifuging, reduction of an alkaline copper sulphate reagent is followed by cooling, and addition of potassium iodide to liberate iodine which then reoxidizes the cuprous copper formed. The amount of iodine thus used up is determined by thiosulphate titration of test and blank and is proportional to the amount of glucose originally present.

**Reagents.** 1. Isotonic sodium sulphate-copper sulphate solution, as for the Haslewood Strookman method, p. 33.

2. Sodium tungstate, 10 per cent. solution of $Na_2WO_4.2H_2O$.

3. Alkaline copper reagent. Prepare as for the Haslewood Strookman method, p. 33, but after adding the Rochelle salt in solution B, wash in 1·0 gram of solid potassium iodate, shake well to dissolve and then make to the mark with water.

4. Potassium iodide, 2 per cent. solution. Prepare freshly.

5. Sulphuric acid, 2 N. Add 5 ml. of the concentrated acid to water and make up to 100 ml. with water.

6. N/400 sodium thiosulphate solution. Prepare freshly by diluting 5 ml. of stock N/10 solution to 200 ml.

7. Starch, 1 per cent. solution.

**Technique.** Place 0·2 ml. of blood directly into 3·5 ml. of the isotonic sodium sulphate-copper sulphate solution. As King *et al.* (1941) have shown, such a mixture can be kept for seventy-two hours without loss of glucose. Add 0·3 ml. of 10 per cent. sodium tungstate solution, mix well and centrifuge.

Add 2 ml. of the alkaline copper reagent to 2 ml. of the supernatant fluid (equivalent to 0·1 ml. of blood) in a 6 × $\frac{3}{4}$ inch test tube and heat in a boiling water bath for ten minutes. Put up a blank at the same time containing 2 ml. of the copper reagent and 2 ml. of distilled water. Plug the tubes lightly with cotton wool. After the heating, cool immediately under running water. Add 1 ml. of the 2 per cent. iodide and then 1 ml. of 2 N sulphuric acid. Allow to stand for one minute and then titrate with N/400 thiosulphate, using the starch as indicator. The blank should require very nearly 11·2 ml. of the thiosulphate.

**Calculation.** The glucose present in 0·1 ml. of blood is proportional to the difference between blank and test. Each ml. of this thiosulphate is equivalent to 0·058 mg. of glucose. Hence

Mg. glucose per 100 ml. blood

$$= (\text{ml. titration of blank} - \text{ml. titration of test}) \times 0{\cdot}058 \times \frac{100}{0{\cdot}1}$$

$$= (\text{ml. titration of blank} - \text{ml. titration of test}) \times 58.$$

**Notes.** (*a*) Check freshly prepared copper reagent against a glucose standard. A 200 mg. per cent. solution is convenient.

(*b*) For blood sugars over 500 mg. per 100 ml. use 1 ml. of supernatant fluid and 1 ml. of distilled water; for the very high sugars over 1,000 mg. per 100 ml. occasionally found in diabetic coma use 0·5 ml. of the supernatant fluid, 1·5 ml. of water and 2 ml. of copper reagent. In diabetic coma use 1 ml. as routine. Make the necessary adjustments in the calculation.

### VI. Maclean's Method (Maclean, 1919)

Proteins are precipitated by means of sodium sulphate, heat, and dialysed iron. Reduction of an alkaline copper solution is used, the amount of reduction being determined iodometrically.

**Reagents.** 1. Sodium sulphate. 15 per cent. solution. Dissolve 150 grams of $Na_2SO_4$. $10H_2O$ in water and make up to 1 litre. It is more convenient to make up large quantities each time, for example 10 litres. Add 0·1 ml. of glacial acetic acid to 100 ml. of this solution before use.

2. Dialysed iron. B.D.H. dialysed iron is suitable.

3. Alkaline copper solution. This contains in each litre of solution:

120 grams potassium bicarbonate.
80 grams potassium carbonate.
3·5 grams copper sulphate ($CuSO_4$, $5H_2O$).
5 grams potassium iodide.
0·5 gram potassium iodate.

Dissolve the bicarbonate and carbonate in about 600 ml. of water with not more than slight warming. Dissolve the copper sulphate in about 100 ml. of water and wash in with constant stirring. Add the iodide and iodate and shake well to dissolve. Make up to the mark with water and mix. The solution keeps well.

4. Sulphuric acid, 25 per cent. (v/v). Add 25 ml. of the concentrated acid carefully to 75 ml. of water.

5. N/400 sodium thiosulphate solution. Prepare immediately before use by diluting 5 ml. of stock N/10 solution to 200 ml.

6. Starch. 1 per cent. solution.

**Technique.** Place 0·2 ml. of blood into 23·8 ml. of the acidified sodium sulphate solution in a 100 ml. conical flask, washing out the pipette by sucking up solution into it several times. Bring just to the boil, cool a little, add 1 ml. of dialysed iron, mix well and filter. To 20 ml. of the filtrate in a 100 ml. conical flask, add 2 ml. of the alkaline copper solution and boil for six minutes on a flame which just brings the mixture to the boil in approximately 100 seconds (95 to 110 seconds). Cool immediately under running water, add 2 ml. of the sulphuric acid, mix, allow effervescence to cease, and titrate with N/400 thiosulphate, using the starch as indicator. To 2 ml. of the copper solution add a few ml. of water and 2 ml. of sulphuric acid and titrate as before. This gives the blank titration and requires 11·2 ml. of thiosulphate.

**Calculation.** The difference between the blank titration and the titration of the test gives a measure of the blood sugar. Subtract accordingly and read off the blood sugar from the Table given, or for blood sugars of more than 150 mg. per cent. calculate from

$$\text{Mg. sugar per 100 ml. of blood} = ((\text{Titration of blank} - \text{titration of test}) + 0{\cdot}1) \times \frac{100}{2}$$

TABLE II

*Blood Sugars. Maclean's Method*

| Titration Ml. (B − T) | Blood Sugar Mg./100 ml. | Titration Ml. (B − T) | Blood Sugar Mg./100 ml. | Titration Ml. (B − T) | Blood Sugar Mg./100 ml. |
|---|---|---|---|---|---|
| 0·1 | 16 | 1·1 | 68 | 2·1 | 114 |
| 0·2 | 22 | 1·2 | 72 | 2·2 | 118 |
| 0·3 | 28 | 1·3 | 77 | 2·3 | 122 |
| 0·4 | 33 | 1·4 | 81 | 2·4 | 127 |
| 0·5 | 38 | 1·5 | 86 | 2·5 | 132 |
| 0·6 | 43 | 1·6 | 91 | 2·6 | 137 |
| 0·7 | 48 | 1·7 | 95 | 2·7 | 142 |
| 0·8 | 53 | 1·8 | 100 | 2·8 | 147 |
| 0·9 | 58 | 1·9 | 105 | 2·9 | 152 |
| 1·0 | 63 | 2·0 | 109 | 3·0 | 156 |

**Notes.** (*a*) For values over 400 mg. per 100 ml. use a smaller amount of the protein-free filtrate. Ten ml. of this plus 10 ml. of 15 per cent. sodium sulphate to bring the volume to 20 ml. is convenient. For values over 800 mg. per 100 ml. use 5 ml. of filtrate plus 15 ml. of sulphate. It is good practice to use 10 ml. filtrate in diabetic coma, repeating with 5 ml. if necessary.

(*b*) Put a complete blank through at intervals to check all of the reagents used. In particular, test each new batch of dialysed iron.

(*c*) The solutions are easy to prepare and keep well. Some workers have found standardizing the flame troublesome.

## VII. Method of Hagedorn and Jensen (1923)

Proteins are precipitated by means of zinc hydroxide and heat. Reduction of alkaline ferricyanide is used, the amount of unreduced ferricyanide being determined iodometrically.

**Reagents.** 1. Zinc sulphate, 0·45 per cent. solution. Prepare a stock solution by dissolving 45 grams of $ZnSO_4$, $7H_2O$ in water and make up to 100 ml. Dilute 1 ml. to 100 ml. once a week.

2. N/10 sodium hydroxide. Keep a stock 2 N solution from which the N/10 solution is prepared weekly.

3. Alkaline potassium ferricyanide solution. Dissolve 1·65 grams of potassium ferricyanide and 10·6 grams of fused sodium carbonate in water, and make up to a litre. Keep in the dark.

4. Iodide-sulphate-chloride solution. Dissolve 10 grams of zinc sulphate, $ZnSO_4$, $7H_2O$, and 50 grams of sodium chloride in water, and make up to 200 ml. Add potassium iodide in the proportion of 0·25 gram per 10 ml. of solution as required. If the iodide is included in the solution (5 grams per 200 ml.) this only keeps for two or three days.

5. Acetic acid, 3 per cent. solution. Dilute 3 ml. of glacial acetic acid to 100 ml. with water.

6. N/200 sodium thiosulphate solution. Prepare before use by diluting a stock N/10 solution.

7. Starch indicator. Use a 1 per cent. solution in saturated sodium chloride solution.

**Technique.** Place 0·1 ml. of blood into a mixture of 1 ml. N/10 sodium hydroxide and 5 ml. of 0·45 per cent. zinc sulphate in a test tube and heat for three minutes in a boiling water bath. Filter through a small filter paper (7 cm. Whatman No. 41) into a 6 × 1 inch test tube, washing out the original tube with 3 ml. of hot distilled water and using this to wash the precipitate. Repeat this washing.

To the whole of this filtrate add 2 ml. of the alkaline ferricyanide solution and heat for fifteen minutes in a boiling water bath. Cool and add 3 ml. of the iodide-sulphate-chloride solution, followed by 2 ml. of 3 per cent. acetic acid. Mix well, stand for a minute or two, and titrate from a microburette with N/200 thiosulphate using 2 drops of the starch solution as indicator.

For the blank, carry out the whole estimation, omitting only the blood. The blank titration should take between 1·80 and 2·00 ml. If it requires less than 1·80 ml. make up fresh ferricyanide reagent.

TABLE III

*Mg. Glucose per* 100 *ml. Blood Equivalent to Ml. N/*200 *Thiosulphate*

(Method of Hagedorn and Jensen.)

| Ml. Thio-sulphate. | 0·00 | 0·01 | 0·02 | 0·03 | 0·04 | 0·05 | 0·06 | 0·07 | 0·08 | 0·09 |
|---|---|---|---|---|---|---|---|---|---|---|
| 0·0 | 385 | 382 | 379 | 376 | 373 | 370 | 367 | 364 | 361 | 358 |
| 0·1 | 355 | 352 | 350 | 348 | 345 | 343 | 341 | 338 | 336 | 333 |
| 0·2 | 331 | 329 | 327 | 325 | 323 | 321 | 318 | 316 | 314 | 312 |
| 0·3 | 310 | 308 | 306 | 304 | 302 | 300 | 298 | 296 | 294 | 292 |
| 0·4 | 290 | 288 | 286 | 284 | 282 | 280 | 278 | 276 | 274 | 272 |
| 0·5 | 270 | 268 | 266 | 264 | 262 | 260 | 259 | 257 | 255 | 253 |
| 0·6 | 251 | 249 | 247 | 245 | 243 | 241 | 240 | 238 | 236 | 234 |
| 0·7 | 232 | 230 | 228 | 226 | 224 | 222 | 221 | 219 | 217 | 215 |
| 0·8 | 213 | 211 | 209 | 208 | 206 | 204 | 202 | 200 | 199 | 197 |
| 0·9 | 195 | 193 | 191 | 190 | 188 | 186 | 184 | 182 | 181 | 179 |
| 1·0 | 177 | 175 | 173 | 172 | 170 | 168 | 166 | 164 | 163 | 161 |
| 1·1 | 159 | 157 | 155 | 154 | 152 | 150 | 148 | 146 | 145 | 143 |
| 1·2 | 141 | 139 | 138 | 136 | 134 | 132 | 131 | 129 | 127 | 125 |
| 1·3 | 124 | 122 | 120 | 119 | 117 | 115 | 113 | 111 | 110 | 108 |
| 1·4 | 106 | 104 | 102 | 101 | 99 | 97 | 95 | 93 | 92 | 90 |
| 1·5 | 88 | 86 | 84 | 83 | 81 | 79 | 77 | 75 | 74 | 72 |
| 1·6 | 70 | 68 | 66 | 65 | 63 | 61 | 59 | 57 | 56 | 54 |
| 1·7 | 52 | 50 | 48 | 47 | 45 | 43 | 41 | 39 | 38 | 36 |
| 1·8 | 34 | 32 | 31 | 29 | 27 | 25 | 24 | 22 | 20 | 19 |
| 1·9 | 17 | 15 | 14 | 12 | 10 | 8 | 7 | 5 | 3 | 2 |

**Calculation.** Read from Table III the mg. glucose per 100 ml. of blood equivalent to the titration of the blank and subtract this from that equivalent to the titration of the unknown. For example, if the titrations of blank and unknown were 1·92 and 1·15 ml. respectively,

$$\text{Mg. sugar per 100 ml. blood} = 150 - 14 = 136.$$

**Notes.** (*a*) This method has been widely used, particularly on the continent of Europe. It is important that the chemicals used in

preparing the solutions should be pure. This is specially so in the case of the ferricyanide which may need to be recrystallized. However, the present-day high grade analytical reagent is usually satisfactory.

(*b*) For high blood sugars it is necessary to use a smaller volume of blood. 0·05 ml. is usually suitable and may be taken as routine in diabetic coma.

### Comparison of Methods

With reasonable care all these methods will give results sufficiently accurate for clinical purposes. In so far as they include varying amounts of non-glucose reducing substances, they will give slightly different normal ranges. The methods in which the blood is placed directly into isotonic sodium sulphate solution (King's modified Schaffer-Hartmann, Haslewood and Strookman, King and Garner), and Benedict's method, give results which may be taken as being near to the true glucose content. Maclean's method also approximates to this figure. The original Folin-Wu method is generally held to give results which may be some 20 mg. per 100 ml. higher than this, which would also seem to be the case with the Hagedorn Jensen method.

Several workers have found the colour to fade. This is more serious when a large number of determinations are being carried out simultaneously. Procedures for stabilizing the colour in the Folin-Wu and Benedict methods have been given. The author has not found appreciable fading in the first half hour when using the Haslewood and Strookman method. King and Garner (1947) studied the changes in colour with time in several methods. Sunderman *et al.* (1951) discuss the relative merits of a number of blood sugar methods.

Of the colorimetric methods the author has used the Haslewood Strookman method most. This, and Benedict's or Somogyi's method, can be used satisfactorily. These are particularly suitable for low blood sugar values. Among the titrimetric methods King's modified Shaffer Hartmann and MacLean's method are convenient and give similar results. The actual choice of method may be influenced by the needs and circumstances of a particular laboratory.

#### INTERPRETATION

The normal fasting or post-absorptive blood sugar, taken not less than three to four hours after the last meal, usually lies between 65 and 120 mg. per 100 ml. With methods giving almost the true sugar it is more likely to be in the lower part of this range between 65 and 100. The commonest finding is in the region of 80 mg. per 100 ml. Higher values are obtained during the period immediately following a meal and will be discussed in the next chapter. Here we are concerned with variations in the fasting blood sugar. Both increase, hyperglycæmia, and decrease, hypoglycæmia, occur in disease.

**Hyperglycæmia.** The highest values for fasting blood sugar are obtained in diabetes mellitus, in which it may vary from normal up

to 500 mg. per 100 ml. and over, according to the severity of the condition. A blood sugar of more than 500 is usually associated with some degree of diabetic coma (see p. 80).

Except in diabetes the fasting blood sugar rarely exceeds 200 mg. per 100 ml. Hyperactivity of the thyroid, pituitary, and adrenal glands, with the latter of which we can include states of emotional stress, may give small increases not exceeding about 150 mg. per 100 ml. There is, however, an increased incidence of diabetes in hyperthyroidism and in hyperpituitarism, when much higher figures are obtained.

In diffuse disease of the pancreas, as in pancreatitis and carcinoma, some increase in fasting blood sugar may occur, but, except in advanced cases, it is moderate and does not much exceed 150 mg. per 100 ml. There has to be extensive disease for sufficient islet tissue to be affected to cause diabetic symptoms. Similar increases, rarely exceeding 160 to 180 mg. per 100 ml., occur in convulsions and in the terminal stages of many diseases.

A rise in blood sugar, which may be quite appreciable, can occur in sepsis and in a number of infectious diseases (see further, p. 48). A moderate hyperglycæmia may also be found in some intracranial diseases such as meningitis, encephalitis, tumours, and hæmorrhage.

The effect of anæsthesia should also be borne in mind. Depending on the degree and duration of the anæsthesia, quite a considerable rise in blood sugar may occur, sometimes to over 200 mg. per 100 ml., thus producing a temporary glycosuria. Asphyxia has a similar effect. These increases may be partly due to the acidosis which is produced and is known to increase glucose formation from glycogen in the liver. Such a factor may also play a part in causing the moderate increase found in terminal states.

One may say that a fasting blood sugar between 150 and 200 mg. per 100 ml. is very suggestive of diabetes mellitus, whilst one of over 200 is almost diagnostic.

**Hypoglycæmia** occurs most frequently as a result of over-dosage with insulin in the treatment of diabetes. Insulin-secreting tumours of the pancreas, which are extremely rare, produce a severe hypoglycæmia, in which glucose may be almost completely absent from the blood. The fasting blood sugar may be reduced in hypothyroidism (myxœdema, cretinism), hypopituitarism (for example, Simmonds' disease), and hypoadrenalism (Addison's disease), with values down towards 20 mg. per 100 ml. in severe cases of the last two of these conditions.

While mild exercise can raise the blood sugar a little, severe exercise may produce a hypoglycæmia due to the liver being depleted of its glycogen, from which the blood sugar is maintained. For the same reason, severe liver disease has a similar effect though low blood sugar levels are not often found. In glycogen storage disease (von Gierke) there is abnormal storage of glycogen, particularly in the liver and heart muscle. This is not available for maintaining the blood sugar level so that a low blood sugar can be found in the post-absorptive period.

There is possibly deficient absorption of glucose in the group of steatorrhœas (see p. 49), but while the blood sugar may be in the lower part of the normal range, it is very rare indeed for it to be subnormal.

The transient post-prandial hypoglycæmia, which may occur in a few people, some one and a half to three hours after taking food and more commonly in patients after partial gastrectomy, will be discussed later (p. 50).

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*CHAPTER III*

# GLUCOSE TOLERANCE TESTS

So far we have been dealing with the fasting blood sugar. Following food, there is a temporary rise in the blood sugar, the extent and duration of which depends on the type of food taken. Carbohydrates mainly cause this increase, which in normal people does not lead to a blood sugar of more than 160 to 180 mg. per 100 ml. There is then a return to normal fasting levels which are reached at the latest within two to three hours after taking the food. This effect of ingested carbohydrate can be studied under reasonably standard conditions by means of the glucose tolerance test, which is thus of considerable use in investigating abnormalities of carbohydrate metabolism, and cases in which glycosuria has been found. The test as commonly carried out will be described first.

## ORAL GLUCOSE TOLERANCE TEST

### Method of Carrying out the Test

The test is usually carried out in the morning after a night's fast, but, if necessary, it may be done some four to five hours after the last food was taken. If the patient is an out-patient coming some distance, a cup of tea without sugar may be allowed on rising.

Blood is taken for the determination of the fasting blood sugar and a specimen of urine is collected. Fifty grams of glucose dissolved in about 150 to 200 ml. of water is then given. This is best followed by about 50 to 100 ml. of water, which takes away the sweet taste and lessens the risk of the patient vomiting, the occurrence of which spoils a small proportion of tests. Blood for estimation of blood sugar is taken at half-hourly intervals for two and a half hours after the glucose has been drunk. Urine specimens are collected at intervals. Some workers collect half-hourly, but it is not always possible to do this. Two specimens, one hour and two hours after taking the glucose can usually be obtained. In many cases it is sufficient to collect a specimen towards the end of the test. This reveals whether any glycosuria has resulted from taking the glucose.

The usual amount of glucose given in this country is 50 grams. Larger amounts have been used, particularly in America, where 1·75 grams per kilogram of body weight has been given. Thirty to 40 grams are usually sufficient to give the maximum rise in blood sugar. Much larger amounts may delay the subsequent fall in the blood sugar, but otherwise the findings are not greatly different.

Smaller amounts should be given to children. Three-quarters of a gram per kilogram of body weight (one-third of a gram per pound) has been recommended. It is convenient and satisfactory to give

20 grams up to about eight years of age, 30 grams up to twelve years, and 40 grams up to fourteen to fifteen years.

**Normal Response.** A typical normal response is shown in Table IV. It is seen that the initial blood sugar is within the normal fasting limits ; the maximum blood sugar, usually between 140 and 180 mg. per 100 ml., is reached either half or one hour after taking the glucose ; the blood sugar then returns rapidly to the normal fasting limits which are often reached in one and a half hours, and almost always by the end of two hours. There is sometimes a slight dip in the blood sugar towards the end of the test as shown in the example given. Finally, there should be no sugar in any of the urine specimens.

TABLE IV

*Normal Glucose Tolerance Curve*

| Time | Fasting | ½ hour | 1 hour | 1½ hours | 2 hours | 2½ hours |
|---|---|---|---|---|---|---|
| | | after taking glucose. | | | | |
| Blood sugar. . | 80 | 155 | 165 | 115 | 75 | 80 |
| Urine sugar . | Absent throughout the test. | | | | | |

**Use of Venous Blood.** The figures given above are for capillary blood such as is obtained from a thumb or finger. Whilst there is little difference between arterial and venous blood sugar at ordinary fasting levels, the arterial blood sugar, and hence the capillary blood sugar, at the peak of the curve, on an average, is 20 to 30 mg. per 100 ml. higher than the venous blood sugar, since there is increased carbohydrate utilization at that time. Accordingly the maximum blood sugar reached in tolerance curves done on venous blood may be lower by that amount.

**Renal Threshold for Glucose.** In normal persons, so long as the blood sugar is below 180 to 200 mg. per 100 ml., glucose in amounts which are detected by the routine tests used, is not excreted by the kidneys. This level of blood sugar has been termed the renal threshold for glucose. As a rough and ready conception this is useful, but too rigid a figure should not be attached to it. For a full discussion, see Peters and Van Slyke (1946). Since the blood sugar does not exceed this threshold in a tolerance test on normal patients, there is then no sugar in the urine.

**The Effect of the Previous Diet.** Special attention must be paid to the type of diet taken in the period before the test is done. If a patient is placed on a low carbohydrate diet for some time before the test, a curve with an appreciably higher maximum, rising to over 200 mg. per 100 ml., can be obtained, so that there may be some glycosuria. Since a person suspected of having diabetes may have been put on such a diet, it is important to see that an ordinary mixed

diet is taken for at least a week before doing the test. Otherwise a curve suggesting a mild diabetes, with a little sugar in the urine, may be obtained. This is most important with patients in whom glycosuria has been found, but who have no symptoms of diabetes. Normal tolerance curves are given by the majority of such cases, particularly by younger people.

**Effect of Age.** The type of curve obtained varies with age. Both the average fasting level and the average maximum to which the blood sugar rises, increase with age, so that in young people they tend to be lower than the example given, whilst in elderly people they are often higher. In old people the maximum blood sugar reached may sometimes exceed 200 mg. per 100 ml. Himsworth (1939) has produced evidence to suggest that sensitivity to insulin is reduced when a person takes a low carbohydrate diet, and tends to fall with increasing age. Such a change would explain the tendency for there to be slightly raised tolerance curves in both these groups of cases.

## Abnormal Tolerance Curves

**Diminished Glucose Tolerance.** This is present when the rise in blood sugar is greater than in normal persons and when the return to normal fasting levels is delayed. In some cases the fasting blood sugar may be higher than the normal limits given. Examples of such curves are given in Table V showing increasing fall in glucose tolerance from columns I to V.

TABLE V

*Diminished Glucose Tolerance*

| | I | II | III | IV | V |
|---|---|---|---|---|---|
| Fasting Blood Sugar (mg. per 100 ml.) | 105 | 115 | 135 | 195 | 235 |
| Blood Sugar ½ hour after 50 grams glucose | 180 | 195 | 215 | 275 | 300 |
| ,, ,, 1 hour after 50 grams glucose | 215 | 255 | 290 | 320 | 345 |
| ,, ,, 1½ hours after 50 grams glucose | 195 | 245 | 285 | 325 | 365 |
| ,, ,, 2 hours after 50 grams glucose | 145 | 185 | 225 | 285 | 355 |
| ,, ,, 2½ hours after 50 grams glucose | 110 | 130 | 170 | 245 | 335 |
| Urine Sugar. Fasting . . . . | — | — | — | + | ++ |
| 1 hour after glucose . . . | tr. | + | ++ | +++ | +++ |
| 2 hours after glucose . . . | tr. | — | ++ | +++ | +++ |

Glucose tolerance is diminished to the greatest extent in diabetes mellitus. Hyperactivity of the thyroid, pituitary, and adrenals may also lead to some degree of impairment, comparable in some cases with that of a mild diabetes. However, there is an increased incidence of diabetes in such endocrinal disorders and then quite severe diabetic curves are obtained. Otherwise the effect on the tolerance curve is usually very moderate. In hyperpituitarism, as found in some stages of acromegaly, the effect is particularly mild, a typical example being shown in column I. Hyperthyroidism can have a greater effect, so that all types of curve from normal up to that

shown in column II may be seen. A decrease in glucose tolerance is also seen in Cushing's syndrome whether due to basophil cell tumour of the anterior pituitary or to an adrenal cortical tumour. This also is mild to moderate, covering the region shown by columns I and II. Injection of adrenocortieotrophic hormone of the anterior pituitary also leads to a diminished tolerance and curves with a maximum blood sugar up to between 250 and 300 mg. per 100 ml. may be obtained. The connection of the adrenal cortex with some rheumatic conditions may explain the finding of mild to moderate impairment of glucose tolerance at some stages of rheumatic disease.

Curves similar to those shown in columns III to V are typical of diabetes mellitus in its more severe forms.

Diffuse disease of the pancreas, if sufficiently severe, may affect enough islet tissue to reduce glucose tolerance temporarily or permanently. However, there is very considerable reserve and even when carbohydrate metabolism is affected in pancreatitis and carcinoma of the pancreas the change in glucose tolerance is usually only moderate.

Very severe liver disease, by reducing the rate of glycogen formation may also lead to tolerance curves in which the blood sugar reaches higher levels than normally. The test, however, is of negligible value in liver disease. Similar curves are obtained in glycogen storage disease. The large amount of glycogen already in the liver makes difficult further formation of glycogen so that the blood sugar rises more than in normal persons.

The influence of infections on glucose tolerance should also be noted. This can be quite considerable. The extent to which tolerance is diminished appears to depend more on the type of infection than on its severity. The effect of staphylococcal infections such as boils and carbuncles is well known. According to Peters even a common cold has been known to produce a temporary glycosuria, yet in some severe infections the effect on glucose tolerance is small. Tolerance usually returns to normal when the infection clears up. The effect of such infections on diabetic patients may be most important since the condition may become much aggravated by them.

**Increased Glucose Tolerance.** Those cases in which there is little rise in blood sugar are said to have an increased glucose tolerance. It has to be admitted that in a number of apparently normal people there is only a small increase in blood sugar which may scarcely exceed the usual normal fasting limits at any time during the test. The most that can be said is that the proportion of such flatter tolerance curves is considerably greater in certain conditions. Examples are given in Table VI.

Such flat curves are frequently obtained in cases in which there is hypoactivity of the endocrines, as in hypothyroidism (myxœdema, cretinism), hypoadrenalism (Addison's disease), and hypopituitarism (Simmonds' disease, Fröhlich's syndrome—dystrophia adiposo-genitalis). The fasting blood sugar may sometimes be below the usual normal limits. However, although flat curves are common in these diseases, there are a number of exceptions.

TABLE VI

*Increased Glucose Tolerance*

| | VI | VII |
|---|---|---|
| Fasting Blood Sugar (mg. per 100 ml.) . . . | 80 | 65 |
| Blood Sugar ½ hour after 50 grams of glucose . . | 105 | 85 |
| ,, ,, 1 hour after 50 grams of glucose . . | 115 | 95 |
| ,, ,, 1½ hours after 50 grams of glucose . . | 105 | 90 |
| ,, ,, 2 hours after 50 grams of glucose . . | 95 | 75 |
| ,, ,, 2½ hours after 50 grams of glucose . . | 85 | 70 |
| Urine Sugar. None throughout. | | |

Similar toleranee curves with little rise in blood sugar are also frequently seen in patients with idiopathic steatorrhœa, cœliae disease and sprue. In these, impaired absorption of earbohydrate has been held to be the eause. The fasting blood sugar is almost always within normal limits.

Because of the overlapping with results obtained in normal persons, such eurves ean only be of limited value but may be signifieant when taken in eonjunction with other findings.

**Renal Glycosuria—Lowered Renal Threshold.** As we saw previously, glueose is not found in the urine of normal people in amounts deteeted by the routine tests used, so long as the blood sugar is below 180 to 200 mg. per 100 ml., the renal threshold for glueose. In a small minority, however, this threshold is redueed and so is lower than the maximum blood sugar whieh may be reaehed in normal persons after taking earbohydrate, whether as a rieh earbohydrate meal or as the glueose in a toleranee test. Sueh persons pass sugar in the urine whenever suffieient earbohydrate is taken temporarily to raise their blood sugar above their lowered renal threshold. The toleranee eurve is normal, though the maximum blood sugar reaehed is sometimes a little lower than in eompletely normal people. Sueh a result is shown in eolumn VIII in Table VII. Carbohydrate metabolism is thus normal, but presumably beeause of some defeet in the tubular reabsorption of glueose, an appreeiable amount of this substanee is allowed to eseape in the urine. The condition is known as *Renal Glycosuria*. As a rule it is diseovered aeeidentally, for example, at medieal examination for life insurance, or when sueh examination is required for eertain oeeupations. A normal toleranee curve is required by the examining body. The eondition is usually symptomless. It is held to be harmless and not likely to develop into diabetes later. The renal threshold also appears to be slightly lowered fairly frequently in the later stages of pregnaney (see further, p. 67).

It is not often necessary to know where the threshold is, but if this information is desired, frequent urine specimens must be eolleeted as the blood sugar is followed. The threshold may oeeasionally be

within the normal fasting limits for the blood sugar, but it is usually somewhere between these and the normal threshold, for example in the region of 140 mg. per 100 ml., so that the glycosuria is intermittent.

**Raised Renal Threshold.** It should also be noted that there are persons in whom the renal threshold is raised. There is a tendency for this to occur with increasing age, and it is also found in quite a number of diabetics. The fact that it appears often to be raised in

TABLE VII

*Glucose Tolerance Curves in Renal Glycosuria, Etc.*

| | VIII | IX | X |
|---|---|---|---|
| Fasting Blood Sugar (mg. per 100 ml.) . . | 85 | 140 | 85 |
| Blood Sugar ½ hour after 50 grams of glucose . | 155 | 215 | 220 |
| ,, ,, 1 hour after 50 grams of glucose . | 135 | 255 | 190 |
| ,, ,, 1½ hours after 50 grams of glucose | 100 | 270 | 115 |
| ,, ,, 2 hours after 50 grams of glucose. | 85 | 240 | 90 |
| ,, ,, 2½ hours after 50 grams of glucose | 80 | 205 | 75 |
| Urine Sugar. Fasting . . . . . | — | — | — |
| 1 hour after taking the glucose . . | + | — | + |
| 2 hours after taking the glucose . . | trace | — | — |

arteriosclerosis is possibly the reason why this is so in many elderly diabetics. As a result a blood sugar of over 200 mg. per 100 ml.—sometimes considerably over—may not produce glycosuria and occasionally persons with diabetes give a tolerance curve reaching to between 250 and 300 mg. per 100 ml. for the blood sugar, without sugar being found in the urine. An example is shown in column IX in Table VII.

**The Lag Type of Tolerance Curve.** Finally there are some apparently normal people who are found to have a tolerance curve in which the blood sugar returns to normal limits in the usual time, but which shows a somewhat exaggerated rise in blood sugar. Because of this the peak of the curve is above the normal renal threshold, so that a transient glycosuria results from taking 50 grams of glucose and may also be produced by a rich carbohydrate meal. An example of such a curve is given in Table VII, column X. This type of curve has been termed a "lag" curve on the assumption that the greater rise in blood sugar is due to a delay in the insulin mechanism coming into action. It is more probably due to an increased rate of absorption of glucose from the intestine, following rapid emptying of the stomach.

This type of glycosuria is also usually discovered accidentally on routine medical examination in the same way as renal glycosuria. Like that condition it is considered harmless and not likely to develop into diabetes later. A similar type of curve with a high peak, some glycosuria, and sometimes with an over vigorous swing back to blood sugar levels below the normal fasting limits, has been observed after

partial gastrectomy. In such cases ingestion of the glucose, or of a meal, may be followed some two to three hours later by symptoms such as dizziness, sweating, a feeling of weakness and nausea, palpitation, due to the rapid fall in blood sugar. The symptoms of hypoglycæmia are rapidly relieved by taking sugar or any easily assimilable carbohydrate. Extended glucose tolerance curves have been used for investigating these and other cases of hypoglycæmia.

### Extended Glucose Tolerance Curves

Instead of ending at two and a half hours after taking glucose, half-hourly blood sugars are done for periods up to four or five hours. Examples of such curves are shown in Table VIII. In column XI is shown a curve from a patient after partial gastrectomy whilst XII

TABLE VIII

*Extended Glucose Tolerance Tests*

| | XI | XII | XIII | XIV |
|---|---|---|---|---|
| Fasting Blood Sugar (Mg. per 100 ml.) . . | 85 | 90 | 88 | 43 |
| Blood Sugar ½ hour after 50 grams glucose . | 190 | 205 | 177 | 110 |
| ,, ,, 1 hour after 50 grams glucose . | 230 | 181 | 167 | 133 |
| ,, ,, 1½ hours after 50 grams glucose . | 135 | 95 | 81 | 123 |
| ,, ,, 2 hours after 50 grams glucose . | 73 | 77 | 65 | 105 |
| ,, ,, 2½ hours after 50 grams glucose . | 58 | 67 | 72 | 91 |
| ,, ,, 3 hours after 50 grams glucose . | 63 | 58 | 77 | 77 |
| ,, ,, 3½ hours after 50 grams glucose . | 73 | 65 | 81 | 67 |
| ,, ,, 4 hours after 50 grams glucose . | 81 | 75 | 91 | 58 |
| ,, ,, 5 hours after 50 grams glucose . | 86 | 85 | — | 48 |

and XIII were found in patients having attacks suggesting hypoglycæmia some two to three hours after food. Both these cases had some degree of hyperacidity and rather rapid emptying of the stomach. However, it must be confessed that in some of these cases the tolerance curves do not fall appreciably below the normal fasting limits (p. 42) for the blood sugar. Yet the attacks can be shown to coincide with the temporary dip in the blood sugar and to be relieved by glucose.

Extended tests are sometimes used to differentiate such transient attacks from those due to insulin-secreting tumours of the islets of Langerhans in the pancreas and other abnormal endocrine conditions such as Simmonds' disease, which cause hypoglycæmia. In these latter conditions the fall in blood sugar at the end of the tolerance test tends to be progressive, as is shown in column XIV, obtained from a case with such an islet tumour. However, the fasting blood sugar, particularly the early morning blood sugar taken before breakfast, is usually particularly low in these cases, and sufficient for diagnosis.

### One Hour—Two Dose Test (Exton Rose)

A form of tolerance test apparently much used in America is the one hour two dose test. Two doses of 50 grams of glucose, separated



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by an interval of half an hour, are given. It is convenient to dissolve 100 grams of glucose in about a pint of water and divide this into two equal parts. The fasting blood sugar is taken and one of these is drunk. After half an hour a second blood sugar is taken and the remaining glucose drunk. After a further half hour a third blood sugar is taken. Urine is collected fasting and at the half hour and one hour. Several approximately similar criteria for assessing the results have been given. The increase in blood sugar should not exceed 70 mg. per 100 ml. in the first half hour, while at the end of an hour the total increase should not exceed 80 mg. per 100 ml. The ordinary glucose tolerance test is more satisfactory.

## INTRAVENOUS GLUCOSE TOLERANCE TESTS

These have been used with the object of avoiding effects arising from defective absorption of the glucose. The amount of glucose most commonly used has been 20 to 30 grams given intravenously over a few minutes.

Soskin (1944), for example, used one-third of a gram of glucose per kilogram of body weight given as a 50 per cent. solution within three to five minutes. Blood sugar estimations were done fasting, and at half-hourly intervals for two hours after the injection. He found that all normal cases required less than sixty minutes for the blood sugar to return to normal, whereas in diabetes, even in mild cases, none took less than 120 minutes to return to the initial level. Soskin also investigated a number of cases of liver disease. None took as long as 120 minutes to return to the pre-injection level but only about 20 per cent. did so in less than sixty minutes. Typical examples are

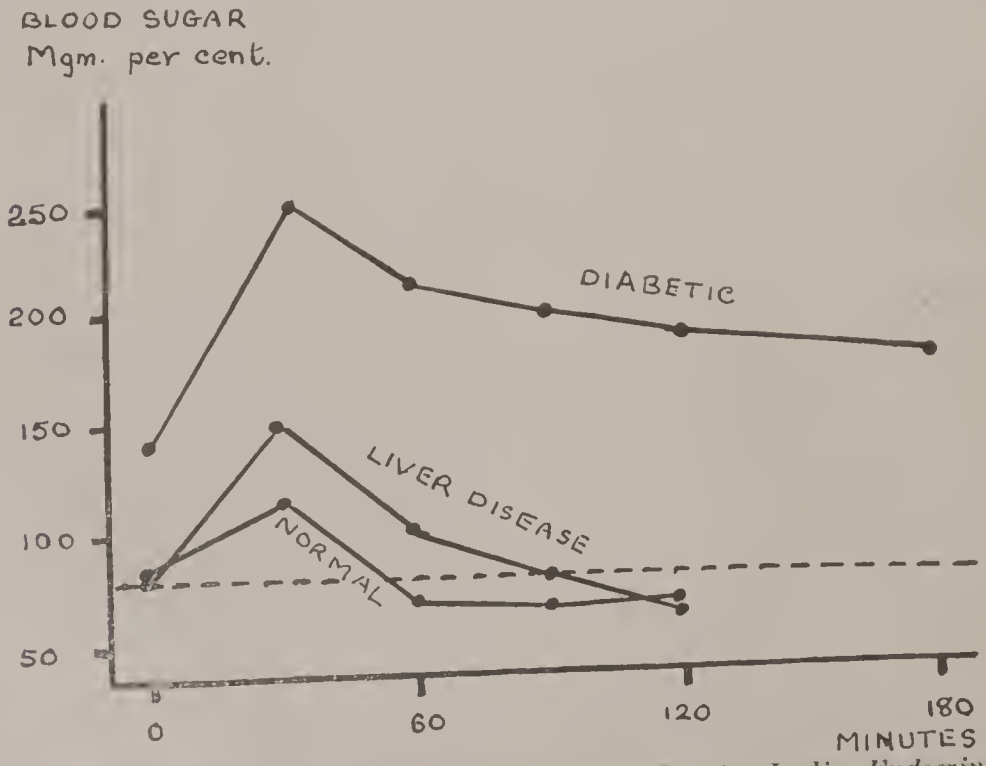


FIG. 14. Intravenous Glucose Tolerance (from Soskin, *J. clin. Endocrinol*, *Charles C. Thomas, Springfield*, 4, 75).

shown in Fig. 14. As with the oral test this was carried out in the morning, no breakfast being allowed.

Whilst its value in liver disease is doubtful, this is a useful form of the intravenous test.

Tunbridge and Allibone (1940) used a fixed amount of glucose irrespective of the body weight, which they found had little influence on the result. These workers gave 92 ml. of a 30 per cent. solution of glucose in distilled water, in three minutes. They used a 50 ml. syringe and chose 92 ml. as convenient for two syringefuls. They estimated the blood sugar at intervals no greater than seven and a half minutes for one hour and found that a normal level was reached in approximately forty-five minutes, ranging from thirty to sixty minutes, a slight hypoglycæmic dip sometimes occurring before the curve finally stayed within normal fasting limits. In disease, variations resemble those found with the oral test, the time to return to normal fasting limits being greater when there is diminished glucose tolerance. It has been asserted that in conditions such as cœliac disease and similar steatorrhœas, which tend to give flat oral tolerance curves, normal results are obtained with the intravenous test and this has been taken to support the view that there is defective absorption in these conditions.

In the very great majority of cases it can be said that there is little to be gained by using the intravenous test rather than the oral one.

### Summary on the Value of Glucose Tolerance Tests

These tests are undoubtedly of most value in the investigation of cases of symptomless glycosuria, such as renal glycosuria and the glycosuria due to "lag" types of curve. They are also of help in recognizing the milder cases of diabetes. They are rarely necessary in severer cases since then the symptoms are almost always quite definite and a single blood sugar is usually sufficient to clinch the diagnosis. They are of little if any value during or in following the course of treatment. The test may contribute useful information in some cases of endocrine dysfunction and in patients with steatorrhœa.

## THE INSULIN TOLERANCE TEST

This test is mainly used in investigating patients with endocrine disorders. The patient should be put on a diet containing at least 300 grams of carbohydrate daily for two to three days before the test is to be carried out. No food is allowed on the morning before the test is done. Blood is taken for the fasting blood sugar. Insulin is then injected intravenously in an amount corresponding to 0·1 unit per kilogram of body weight. Further blood sugars are taken 20, 30, 45, 60, 90 and 120 minutes after the insulin was given.

The normal response is a fall of the blood sugar to approximately 50 per cent. of the fasting level in about half an hour, followed by a steady rise back to the normal fasting limits which are reached within 90-120 minutes. An example is given in column I, Table IX.

TABLE IX

*Insulin Tolerance Tests*

| | I | II | III | IV |
|---|---|---|---|---|
| Fasting Blood Sugar (mg. per 100 ml.) . . | 80 | 80 | 65 | 70 |
| Blood Sugar 20 minutes after insulin . . | 45 | 65 | 35 | 35 |
| ,, ,, 30 minutes after insulin . . | 38 | 70 | 25 | 30 |
| ,, ,, 45 minutes after insulin . . | 48 | 75 | 25 | 30 |
| ,, ,, 60 minutes after insulin . . | 60 | 80 | 25 | 40 |
| ,, ,, 90 minutes after insulin . . | 75 | 85 | 25 | 45 |
| ,, ,, 120 minutes after insulin . . | 85 | 90 | 25 | 55 |

Fraser, Albright and Smith (1941), who suggested this test, recognize two types of abnormal response :

1. The *insulin resistant type*, in which there is a relatively slight or delayed fall in blood sugar. This may be obtained in some cases of diabetes mellitus, in adrenal cortical hyperfunction (Cushing's syndrome), and in anterior pituitary hyperfunction (acromegaly). Results of this type may also sometimes be obtained in the earlier stages of rheumatoid conditions. An example is given in column II of Table IX.

2. *Hypoglycæmia Unresponsiveness*, in which the blood sugar falls as in the normal person, or to even lower levels, but in which the subsequent rise is delayed or even does not occur. This is seen particularly in anterior pituitary hypofunction (Simmonds' disease) ; in adrenal cortical hypofunction (Addison's disease) ; and in hyperinsulinism. It may be useful in distinguishing hypopituitarism from anorexia nervosa. In hypothyroidism the return to a normal blood sugar occurs more slowly than in normal persons (see column IV in the table). In Simmonds' disease in particular, the blood sugar may show no sign of beginning to rise again even at the end of the test. An example of such a finding is shown in column III. In such cases it may be rather dangerous to give even the small dose of insulin mentioned. So in cases of suspected Simmonds' disease the dosage should be reduced to a half or a third of the amount stated, and the patient watched carefully for signs of hypoglycæmia. It is often advisable to give such patients glucose at the end of the test, sometimes intravenously.

The insulin tolerance test may also be used to differentiate insulin resistant or insulin insensitive diabetics from those who are normally sensitive to insulin and whose diabetes is due to a deficient production of insulin (see p. 71). For this purpose Himsworth (1939) used a *Glucose—Insulin Test* in which glucose and insulin are given simultaneously, the insulin intravenously in the same amount as in the insulin tolerance test, the glucose by mouth as in the glucose tolerance test or as Fraser has suggested, 0·1 unit of insulin and 0·8 gram of glucose per kilogram of ideal body weight. (This is the weight a person should have for his or her height and age. See Appendix, p. 518.) In insulin sensitive persons there is little alteration in the

blood sugar after these two injections whilst the blood sugar rises if the patient is insulin insensitive.

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*CHAPTER IV*

# TESTS FOR GLUCOSE AND OTHER REDUCING SUBSTANCES IN URINE

Tests for reducing substances in urine are usually made with the object of deciding whether or not glucose is present. Of such tests the two most commonly used are those of Benedict and Fehling, in which alkaline copper solutions are reduced to cuprous oxide. Although normal urines are able to give a slight reduction when blood sugar methods are applied to them, this is not sufficient to show a positive result with these tests.

## Benedict's Test

**Benedict's qualitative solution** is used. This is prepared as follows : Dissolve with heat, 173 grams of sodium citrate and 100 grams of anhydrous sodium carbonate in about 600 ml. of water in a beaker. Into this run slowly, with constant stirring, a solution of copper sulphate ($CuSO_4$, $5H_2O$) containing 17·3 grams dissolved in about 100 ml. of water. Cool, transfer to a litre volumetric flask, and make to the mark with water.

**Technique.** Add 8 to 10 drops (or 0·5 ml.) of urine to 5 ml. of the reagent in a test tube, and boil for two minutes or place in a boiling water bath for five minutes. Allow to cool spontaneously. A precipitate forms, varying from greenish to yellowish brown to reddish brown, roughly according to the amount of reducing substance present. A white precipitate may be produced if considerable amounts of phosphates are present. Positive results are obtained if reducing substances equivalent to more than 0·10 to 0·15 per cent. of glucose, by blood sugar methods, are present.

## Fehling's Test

**Fehling's Solution.** Prepare freshly at frequent intervals by mixing equal volumes of two stock solutions, a copper solution A, and an alkaline tartrate solution, B.

Solution A. 6·93 grams of $CuSO_4$, $5H_2O$ per 100 ml. of solution.

Solution B. 20 grams of potassium hydroxide and 34·6 grams of sodium potassium tartrate (Rochelle salt) per 100 ml. solution.

**Technique.** Place equal volumes of Fehling's solution and urine in two separate test tubes and bring both to the boil. The Fehling's solution should give no precipitate on boiling. When boiling, add the urine to the Fehling's solution and observe. The mixture may be divided into two portions and once again brought to the boil, but continued heating should not take place. The amount and colour

of the precipitate give a rough idea of the amount of reducing substance present. Small amounts give a greenish-yellow colour, larger ones a yellow to deep reddish brown. The colour, however, is influenced by other factors, such as the rate at which the urine is added to the hot alkaline copper solution.

Benedict's test is the more satisfactory. It is less likely to give weakly positive results with concentrated urines, due to the action of creatinine and uric acid, and it is rather more sensitive to small quantities of glucose because of the weaker alkali it contains. The strong alkali of the Fehling's solution can polymerize small amounts of glucose, particularly if the mixed solutions are vigorously boiled.

**Clinitest.** This is a useful variant of the above reducing tests, particularly suitable for tests made away from a laboratory either by physicians or patients. The alkaline copper reagent is made up in tablet form. The tablets contain copper sulphate, caustic soda (38·5 per cent.), sodium bicarbonate, and citric acid. A small test tube and dropper are provided with the outfit. Measure 5 drops of urine and 10 drops of water into the tube and add one tablet. Owing to the large amount of caustic soda present, sufficient heat is generated to give rapid reduction. A small colour chart is included, so that a rough assessment of the amount of sugar present can be made. Originated by the Ames Company, Inc., Indiana, U.S.A., the sole distributors for the British Isles are Don S. Momand, 57 Albany Street, London, N.W.1. The outfit is stocked by some chemists.

### Nylander's Test

Another test, not often used, is Nylander's, in which urine is heated with one-tenth of its volume of Nylander's reagent (warm 2 grams of bismuth subnitrate and 4 grams of Rochelle salt with 100 ml. of 10 per cent. sodium hydroxide, cool, and filter) in a boiling water bath for five minutes. The bismuth is reduced to the metallic form which appears as a black precipitate. This test is not given by creatinine and urates, but it is necessary, before carrying out the test, to remove any proteins present by boiling and filtering.

## NATURE AND IDENTIFICATION OF REDUCING SUBSTANCES IN URINE

A number of substances which reduce the above solutions are found in urine. These include the carbohydrates : glucose, fructose, lactose, galactose, pentoses ; glucuronates, formed usually by linkage of drugs with glucuronic acid ; salicyluric acid ; homogentisic acid ; uric acid and creatinine, when present in considerable amounts in concentrated urines.

It is sometimes necessary to determine the nature of the reducing substance present. The two general tests most used have been the fermentation test with yeast and the preparation of osazones. Recently paper chromatography has been applied to this problem. In addition, a number of special tests for individual reducing substances are available.

## Fermentation Test

A simple form of apparatus is shown in Fig. 15, and consists of a small beaker and test tube of convenient size. Mix a few grams of washed yeast with 25 to 30 ml. of the urine, which has been previously boiled and cooled. Fill the test tube with this, cover with a finger, invert and slip under the remaining liquid in the beaker. (Alternatively tubes such as those shown in Fig. 15 may be used. Introduce the urine-yeast mixture so that the closed limb is filled.) For reliable results the yeast must be washed to remove any sugar which may be present, and the urine boiled to drive off any dissolved gases,

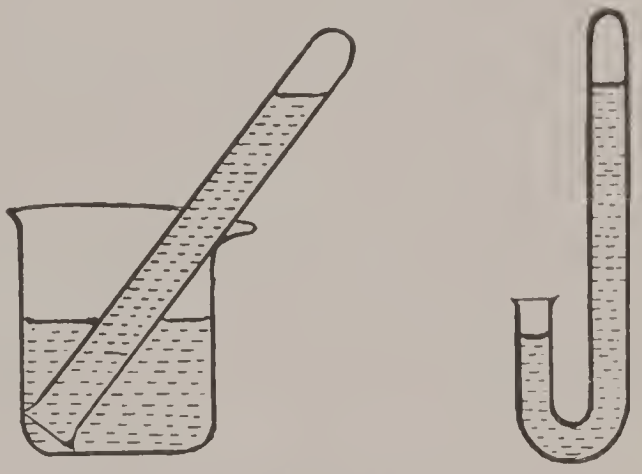


Fig. 15. Apparatus for Fermentation Tests.

and destroy micro-organisms (*B. coli* can form gas from lactose). To wash the yeast, mix well with distilled water, and either centrifuge or filter on the Buchner funnel. Put up positive and negative controls using a normal urine, boiled and cooled, both with and without added glucose. Place the tubes in an incubator at 37° C. Fermentation will occur at room temperature, but is more rapid in the incubator. Production of gas is obvious in an hour or so and should be complete in twenty-four hours when the mixture can be tested again for the presence of reducing substances.

**Significance.** The test is positive only with glucose and fructose.

## Osazone Test

**Technique.** Make 5 to 10 ml. of urine just acid to litmus with acetic acid and mix in a test tube with half a gram to a gram of a mixture of crystalline sodium acetate and phenylhydrazine hydrochloride in the proportion of two parts to one. Place in boiling water for half to one hour and allow to cool slowly in the water after removing the burner. Examine the deposit microscopically.

Phenylglucosazone, which is formed from glucose, fructose and glucuronates, is easily obtained as a rule and forms sheaves of needle-like crystals (Fig. 16). Phenyllactosazone crystals, however, are obtained in most cases only with difficulty with the amounts of lactose which are usually present in urines containing it. They are smaller

FIG. 16. Glucosazone Crystals.

than the glucosazone crystals and more feathery in appearance (Fig. 17). Small atypical osazone crystals with negative fermentation test are most probably lactosazone crystals. Pentosazone crystals are more like glucosazone crystals but are usually much smaller.

For the attempted preparation of lactosazone crystals Cole recommends adsorption on to charcoal by boiling, followed by solution out into 10 per cent. acetic acid with subsequent preparation of osazones.

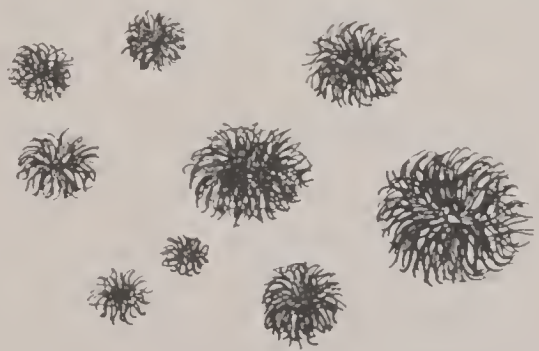


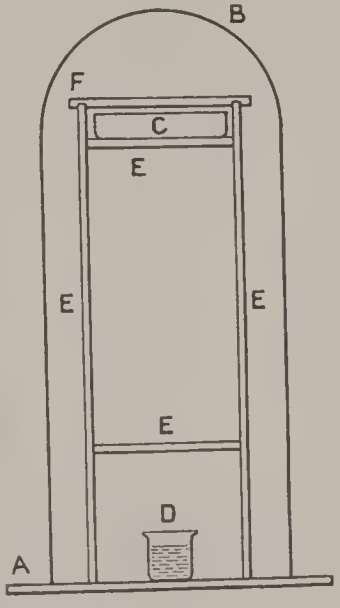
FIG. 17. Lactosazone Crystals.

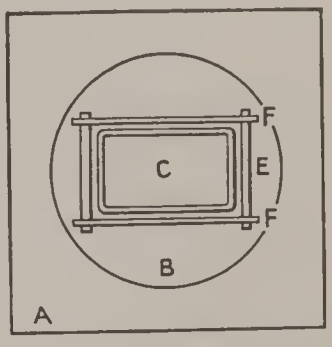
Mix 25 ml. of urine with 1 gram of charcoal and boil for a few seconds. Cool and shake at intervals for ten minutes. Filter, drain thoroughly, and transfer the charcoal to a mixture of 10 ml. of water and 1 ml. of glacial acetic acid in a porcelain basin. After boiling for ten seconds filter into a boiling tube containing about half to a gram of the mixture of sodium acetate and phenylhydrazine hydrochloride used above. Mix well and place the tube in boiling water for forty-five minutes. Allow to cool spontaneously in the water for at least an hour. Examine any deposit under the microscope.

## Partition Chromatography

Separation of carbohydrates by paper chromatography has been worked out by Partridge (1946, 1948), Partridge and Westall (1948), and Flood *et al.* (1947, 1948), and applied to the identification of reducing substances in urine by Horrocks and Manning (1949).

**Reagents.** 1. Solvent. *n*-butyl alcohol—acetic acid—water mixture (Partridge and Westall, 1948). Mix 40 ml. *n*-butyl alcohol with




A. Plate-glass base.
B. Bell jar.
C. Trough containing solvent.
D. Beaker containing water saturated with solvent.
E. Wooden frame.
F. Glass rod.
P. Filter-paper cylinder.

Fig. 18. Apparatus for Descending Paper Chromatography of reducing Substances in Urine. (*The Lancet.*)

10 ml. glacial acetic acid and 50 ml. of distilled water. Shake well in a separating funnel, allow to separate, and run the aqueous layer into a beaker, which is then stood inside the chromatographic apparatus. Run the butyl alcohol layer into the trough of the apparatus (see below).

The following spraying agents are recommended.

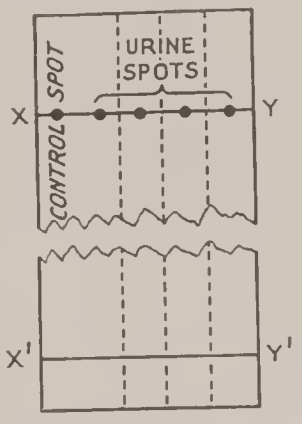
2. Benzidine reagent. 0·5 gram benzidine, 20 ml. glacial acetic acid and 80 ml. absolute ethyl alcohol.

3. Phloroglucinol reagent. Dissolve 0·2 gram phloroglucinol in 80 ml. of 90 per cent. ethyl alcohol and make up to 100 ml. with 25 per cent. w/v trichloracetic acid solution.

4. Ammoniacal silver nitrate. Prepare freshly for use by mixing equal parts of N/10 silver nitrate and 5 N ammonia solution. Discard any reagent not used since highly explosive compounds may form on standing.

5. Aniline hydrogen oxalate reagent. Shake 0·9 ml. of redistilled aniline with 100 ml. of M/10 oxalic acid solution.

**Technique.** Horrocks and Manning carried out the test inside a tall bell jar standing on a glass plate. Their apparatus is shown in Fig. 18. Take a piece of Whatman No. 1 filter paper about 50 cm. long, and of such a width that it can fit in the trough. Draw a pencil line parallel to one end and distant about 10 cm. from it. Place at about 2 cm. intervals along this line, 4 drops of urine and 10 c. mm.




Cut into strips along dotted lines after drying.
X Y line where drops urine applied.
C. Control spot. U. Urine spots.
X′Y′ Solvent point.

Fig. 19. Filter Paper Strips for Descending Chromatography. (*The Lancet.*)

of a control solution containing about 1 per cent. glucose and 1 per cent. lactose. A 20 c. mm. hæmoglobin pipette subdivided into approximately 5 and 10 c. mm. is convenient. The volume of urine used varies with the amount of reducing substance present. Use 5 c. mm. if more than 1 per cent. is present, 10 c. mm. for 0·2 to 1 per cent., 20 c. mm. for less than 0·2 per cent. Dry the filter paper at room temperature or in a low temperature oven. If 20 c. mm. is used, apply in two 10 c. mm. drops, drying the first spot before adding the second. For descending chromatography hold the end of the filter paper in the trough with a glass rod or a microscope slide. The line X . . Y then hangs parallel to and about 1 cm. below the glass rod F, which should be about 3 cm. above the level of the solvent in the trough. For ascending chromatography, form the filter paper into a cylinder and stand upright in the trough. A 4-inch petri dish can be used and will accommodate a strip of filter paper 28 cm. wide, on which fourteen spots can be placed. Fill the trough with the solvent and place the beaker containing the aqueous butyl alcohol-acetic acid

phase on the glass base. Allow the solvent to flow until the solvent front has travelled 30 cm. or more, which may require twenty-four hours or longer. Remove the filter paper, blot between filter paper sheets to remove excess of solvent, and outline the solvent front in pencil.

Now spray rapidly and evenly. An atomizer can be used. Remove excess of reagent by blotting between filter papers and place the sprayed paper in an oven at 100° to 105° C. for ten to twenty minutes according to the reagent used. Observe at five-minute intervals. It is convenient before spraying to cut the filter paper into strips as shown in Fig. 19. The strips can then be sprayed with different reagents. Compare the position of any reducing substances present in the urine, with controls. The term $R_F$ is used, and is given by

$$R_F = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by advancing solvent front.}}$$

This is characteristic for a particular reducing substance in a particular solvent. A correction can be made for temperature by

TABLE X

*$R_F$ Values for Solvent n-Butyl Alcohol, Acetic Acid, Water*

| | I | II | III |
|---|---|---|---|
| Lactose | 0·05 | 0·09 | 0·06 |
| Maltose | 0·07 | 0·11 | 0·06 |
| *d*-Glucuronic acid | — | 0·12 | — |
| *d*-Galacturonic acid | — | 0·14 | — |
| *d*-Galactose | 0·17 | 0·16 | 0·12 |
| *d*-Glucose | 0·19 | 0·18 | 0·15 |
| *l*-Sorbose | — | 0·20 | — |
| Sodium urate | 0·20 | — | 0·17 |
| *d*-Arabinose | 0·22 | 0·21 | 0·21 |
| *d*-Fructose | 0·22 | 0·23 | 0·19 |
| Dehydroascorbic acid | — | 0·27 | — |
| *d*-Xylose | 0·28 | 0·28 | 0·25 |
| *d*-Ribose | 0·29 | 0·31 | — |
| *d*-Xyloketose | 0·32 | — | — |
| *l*-Ascorbic acid | 0·45 | 0·38 | 0·42 |
| Homogentisic acid | 0·85 | — | 0·82 |
| Urine containing salicylates | 0·87 | — | — |

I. Descending chromatography. Horrocks and Manning, not corrected for temperature.

II. Descending chromatography. Partridge and Westall (1948), corrected to 20° C.

III. Ascending chromatography. Horrocks and Manning, not corrected for temperature.

## Table XI

*Colour Reactions for Paper Chromatography of Reducing Substances in Urine*

| Reducing Substance | Spraying Agent | | | |
|---|---|---|---|---|
| | Benzidine | Ammoniacal Silver | Aniline hydrogen oxalate | Phloroglucinol |
| Lactose | Brown (ten minutes). | Light brown or orange. | Yellow. | Nil. |
| Maltose | Brown (ten minutes). | Light brown. | Nil. or faint yellow. | Nil. |
| Galactose | Brown (ten minutes). | Brown. | Brown (ten minutes). | Faint brown. |
| Glucose | Brown (ten minutes). | Brown. | Brown (ten minutes). | Nil. |
| Fructose | Brown (ten minutes). | Light brown. | Faint yellow. | Orange brown. |
| Pentoses | Chocolate brown, developing rapidly within five minutes. | Black or dark brown, developing slowly in the cold. | Bright red spot, developing rapidly within five minutes. | Greenish blue, developing slowly, increasing in intensity on standing. |
| Ascorbic Acid | Pale brown spot. | Black spot develops rapidly on warming or within a minute or so at room temperature. | Nil or faint yellow (fifteen minutes). | Nil. or faint yellow. |
| Urates | Nil or very faint brown. | Black spot develops rapidly on warming or slowly at room temperature. | Nil. | Nil. |
| Homogentisic Acid | Nil. | Intense black spot appears instantaneously on spraying. | Nil. | Nil. |
| Urine containing salicylates. | Nil. | Brown spot only on heating at 100°–105° C. | Nil. | Nil. |

using as standard, glucose at different temperatures. Multiply the observed $R_F$ by $a_t$,

$$a_t = \frac{R_F \text{ for glucose at } 20^\circ \text{ C.}}{R_F \text{ for glucose at } t^\circ \text{ C.}}$$

The nature of a reducing substance can be determined by its $R_F$ value, Table X, and its behaviour with spraying reagents, Table XI. Spray the strip containing the control spot and a urine spot first with benzidine. This will detect 5 μg. of reducing sugars. If a hexose is present spray another strip with the phloroglucinol reagent. This will detect less than 5 μg. of fructose and about 20 μg. of pentoses. If the benzidine test shows a pentose, confirm with the aniline hydrogen oxalate spray. This will detect less than 5 μg. of pentoses, 5 μg. of glucose and galactose, and 10 μg. of lactose and fructose. If no colour is given by benzidine on the first strip, no sugar is present. Spray a further strip with the ammoniacal silver nitrate reagent.

### Polarimetry

Additional information can be obtained from the polarimeter if the laboratory possesses one, though with small amounts of reducing substance this instrument is not very useful. Glucose and lactose are dextrorotatory, fructose lævorotatory, pentoses variable, glucuronates lævo but free glucuronic acid dextrorotatory.

There are a number of *special tests* for these reducing substances which may be useful.

### Seliwanoff's Test for Fructose

Hydrochloric acid acts on fructose to form a derivative of furfuraldehyde which gives a red-coloured compound when linked with resorcinol.

**Seliwanoff's Reagent.** Dissolve 50 mg. of resorcinol in 33 ml. of concentrated hydrochloric acid and dilute to 100 ml. with water.

**Technique.** Add a few drops of urine (about 0·5 ml.) to about 5 ml. of the reagent in a test tube and bring to the boil. Fructose gives a red colour within half a minute. Contrast if necessary with a normal urine. The test is sensitive to 0·1 per cent. if glucose is absent, but if glucose is present it is less sensitive and in addition large amounts of glucose can give a similar colour.

### Bial's Test for Pentoses

Hydrochloric acid acts on pentoses, in a way comparable to its action on fructose, to yield substances which link with orcinol to form green compounds.

**Bial's Reagent.** Dissolve 300 mg. of orcinol in 100 ml. of concentrated hydrochloric acid and add 5 drops of 10 per cent. ferric chloride solution.

**Technique.** Mix 5 ml. of Bial's reagent with 0·5 ml. of the urine and heat until the liquid begins to boil. Pentoses give a green colour.

Fructose, as with Seliwanoff's reagent gives a red colour. The test is sensitive to 0·1 per cent. of pentoses. Glucuronates can give a similar colour if the boiling is prolonged.

### Tauber's Test for Pentoses (Tauber, 1937)

Add 1 ml. of 4 per cent. benzidine solution in glacial acetic acid to 0·2 ml. of urine. Heat until the mixture boils vigorously. Cool under running water and add 2 ml. of water. A pink to red colour is given by pentoses. If large amounts of protein are present remove by mixing equal volumes of urine and 10 per cent. trichloracetic acid and filter. Do the test on the filtrate. The test is very specific for pentoses.

### Mucic Acid Test for Lactose and Galactose

Evaporate 12 ml. of concentrated nitric acid and 50 ml. of urine down to about 10 ml. on a water bath. Cool, add 10 ml. of water and allow to stand overnight. A fine white precipitate is obtained if lactose or galactose is present.

### Methylamine Test for Lactose (Fearon, 1947)

Add 1 ml. of a 0·2 per cent. solution of methylamine hydrochloride in water, followed by 0·2 ml. of 10 per cent. sodium hydroxide, to about 5 ml. of urine. Mix by inversion, cover the tube with a glass bulb and heat at 56° C. for thirty minutes. Remove from the bath and stand at room temperature. Urines containing much lactose will show a red colour before the end of the heating. Colour increases on standing reaching a maximum in about an hour, so that urines low in lactose are best observed after standing. Compare with a blank, particularly if there is only a small amount present. 0·5 per cent. lactose gives an intense red colour after fifteen to twenty minutes at 56° C., 0·05 per cent. a slight but definite colour after standing thirty minutes at room temperature. The only sugars which give a red colour are reducing disaccharides, lactose and maltose. Glucose, fructose, xylose, galactose and sucrose in large amounts give a yellow colour which is easily distinguished.

### Tollen's Test for Glucuronic Acid

Add 0·5 to 1·0 ml. of 1 per cent. naphthoresorcinol in 95 per cent. alcohol and 5 ml. of concentrated hydrochloric acid to 5 ml. of urine. Heat gently to boiling and boil for one minute with constant shaking. Cool slowly. Extract with an equal volume of ether. Glucuronates give a violet or red colour in the extract. Pentoses give a similar colour.

### Tollen's Test for Pentoses, Galactose, and Glucuronic Acid

Add a little phloroglucinol to a mixture of equal volumes of urine and hydrochloric acid. Heat on a boiling water bath. Galactose, pentoses, and glucuronic acid give a red colour, but whereas pentoses and glucuronic acid give a band in the yellow on spectroscopic

examination, galactose does not. This test is given by galactose but not by lactose.

### Test for Salicyluric Acid

Salicylurie acid if present as a result of taking salicylates can be recognized by the ferrie chloride test for salicylates. Add 10 per cent. ferrie chloride drop by drop to a few ml. of urine in a test tube. Salicylates give a reddish purple colour, which is not affected by boiling. A precipitate of phosphates may form at first, but further addition of ferric chloride gives the typical colour.

**Homogentisic Acid.** See pp. 68, 490, for tests for this.

### Significance of Reducing Substances in Urine

**Glucose.** Except for the very rare cases of galactosuria referred to below, glucose is the only one of the carbohydrates found in urine which is significant pathologically. As we have seen, glucose is present in the urine whenever the blood glucose rises above the renal threshold. So long as the threshold is normal, glycosuria is found in all conditions in which the glucose tolerance is diminished.

The most important of these is *diabetes mellitus*, in which there may be as much as 5 to 6 per cent. glucose in the urine. Glucose is also found in the urine of patients with hyperactivity of the endocrines, that is, in *hyperthyroidism*, in *hyperpituitarism*, and in *hyperadrenalism* and may also be produced by injection of the hormones of these glands. The amount of glucose found in these conditions is usually only slight to moderate. In this connection, severe emotional disturbances, produced, for example, by anger, fear, or excitement, may lead to a temporary glycosuria as a result of the secretion of adrenaline. *Severe liver disease* and *whole organ disease of the pancreas* (as distinct from disease of the Islets of Langerhans causing diabetes) may also be accompanied by some glycosuria. As seen earlier (p. 48) there is diminished glucose tolerance in many *infections* and in a number of terminal conditions so that glucose may sometimes be found in the urine in these states. It should also be noted that anæsthesia causes a rise in blood sugar, sufficiently great, in some instances, to exceed the threshold, so that glycosuria may result from *anæsthesia* in normal persons. *Asphyxia* has a similar effect.

Finally there are those cases of apparently normal people, who show a rather exaggerated response to carbohydrate, giving a transient glycosuria, and those cases of *renal glycosuria*, in which glucose tolerance is normal, but the renal threshold is lowered. Whilst usually small in amount, considerable quantities of glucose may be excreted in some cases.

Except in the case of severe diabetes mellitus the glycosuria is likely to be intermittent.

**Lactose.** This is the most important of the other carbohydrates which may be present in urine. It is sometimes found in the urine of women during lactation, and occasionally towards the end of pregnancy. The sudden stopping of lactation is said to be particularly

likely to cause a temporary lactosuria. The condition is naturally a harmless one.

The question of a positive test for reducing substances in the later stages of pregnancy is one which is of some importance. The majority of such tests show the presence of only a small amount of reducing substance, and in most cases this is found to be glucose, the condition being apparently a renal glycosuria. glucose tolerance being normal. There are thus three possibilities if sugar is found in the urine during pregnancy. These are (1) glucose, the result of a renal glycosuria ; (2) glucose, arising from a true diabetes mellitus ; and (3) lactose. Of these the first is by far the commonest, and only the second is of serious significance. In investigating such a case the more common procedure is to do a blood sugar determination. If this is normal, the glycosuria slight, and the condition symptomless, it may not be considered necessary to proceed further. But if any doubt remains or one wishes to be more certain a glucose tolerance test will settle the question. If this is normal the condition is a harmless one and the actual nature of the reducing substance one of interest only.

**Galactose.** Galactose is found in the urine only very rarely. It may be excreted after taking considerable amounts of galactose or lactose. It has been said to occur occasionally in lactation. A galactosæmia leading to a galactosuria has been reported in infants, due to an inability to metabolize galactose (see Bray, Isaac, and Watkins, 1952 ; Donnell and Laun, 1951 ; Townsend, Mason. and Strong, 1951).

**Fructose.** Fructose may be found in the urine after foods which are rich in it, have been eaten. These are mostly fruits, honey, syrup, and jams. It may also be found along with glucose in the urine of diabetics. Fructosuria also occurs, though very rarely, as a congenital defect—essential fructosuria. In no case is the condition harmful but should not be wrongly assumed to be diabetic.

**Pentoses.** These occur under similar conditions to fructose, that is, after eating considerable amounts of certain fruits, such as damsons, cherries, prunes, plums, and as a congenital abnormality. Again, in neither case is the condition harmful, but as with fructose it is important that the case should not be wrongly treated as diabetic. Pentoses may also occur along with glucose in the urine of diabetics.

Galactose, fructose and pentoses have no renal threshold so that they are excreted in the urine as soon as there is an appreciable amount present in the blood.

**Glucuronates.** Present only in traces in normal urine these are excreted in greater amounts when certain drugs are being taken. It is then that alkaline copper solutions may be reduced. The drugs are conjugated with glucuronic acid. Examples are antipyrene, sulphonal, menthol, morphine, salicylic acid, chloral hydrate. If the urine is allowed to stand, glucuronic acid is liberated and can reduce the copper solution. As we have seen, they are not fermented by yeasts and certain distinguishing tests can be used. Furthermore, if the drug is withdrawn the tests should become negative.

**Salicyluric Acid.** This substance, formed by the conjugation of glycine with salicylic acid, may be excreted when salicylates are being taken. Tests, as with glucuronates, become negative on withdrawing the drug.

**Homogentisic Acid.** This is found in the urine in the very rare condition, alkaptonuria, in which metabolism of phenylalanine and tyrosine is incomplete. This is discussed more fully later (see p. 490). The urine, on standing, gradually darkens in colour from the surface downwards, owing to the oxidation of the homogentisic acid. A greenish-brown or black colour results from boiling such a urine with Benedict's solution. The precipitate appears brown when formed, but when it settles to the bottom of the tube it is seen as yellow cuprous oxide. Heated with Fehling's solution, the urine becomes black, and a reddish precipitate settles out. Yeast does not ferment, and there is no rotation of the plane of polarized light.

**Uric Acid and Creatinine.** In high concentration these substances may give slight reduction. This is more likely to occur with Fehling's solution than with Benedict's.

## Investigation of a Case in which there is a Positive Test for Reducing Substance in the Urine

It is clear from what has been said, that with rare exceptions only glucose of the many substances which can reduce alkaline copper solutions, has pathological significance. Then the only point of importance is to decide whether the patient has diabetes mellitus. If the symptoms of diabetes are present, the diagnosis is confirmed either by a blood sugar estimation or by a glucose tolerance test in those cases in which the blood sugar is within normal limits.

An important group of cases is that in which a positive test has been obtained on a routine medical examination, as in pregnancy, for life insurance, etc., there being no symptoms suggesting diabetes. In the majority of such cases the urine contains glucose, but only rarely do the persons have diabetes. As the important point is to exclude diabetes, the tendency nowadays is to do a glucose tolerance test, and, if that is normal, to ignore the positive reduction test as of no significance and not to investigate the nature of the reducing substance present in the urine.

If, however, it is desired *to determine the nature of the reducing substance the following procedure may be used.*

1. Carry out the fermentation test with yeast. This is the most valuable simple test. A positive result indicates the presence of glucose or fructose, or both. Test for fructose with Seliwanoff's reagent. If this is negative glucose is present.

If the fermentation test is negative, glucose is excluded and in almost all cases (see above) the reducing substance can be ignored.

After yeast treatment, test the urine with Benedict's or Fehling's reagent to find whether any other reducing substances are present.

2. Carry out paper chromatography. This is particularly valuable for the sugars which occur less commonly and when more than one is present.

These investigations are usually sufficient, but special tests can also be done.

3. Test for salicylates with a few drops of ferric chloride.

4. If a nursing mother or infant test for lactose and galactose by special tests. Osazones may be prepared.

5. Test for pentoses, for example, by Bial's test.

6. Test for glucuronates by special tests and see if the patient has been taking any drugs likely to form them.

7. If everything else is negative, note whether the urine is a concentrated one.

## QUANTITATIVE DETERMINATION OF GLUCOSE IN URINE

For most purposes a sufficiently good idea of the amount of glucose can be obtained from the qualitative tests. Thus with Benedict's test, it is useful to remember that with urines containing 2 per cent. sugar or more, the supernatant fluid is colourless when the precipitate is allowed to settle out. A greenish precipitate is obtained with sugar in the region of 0·25 per cent., a greenish-yellow at about 0·5 per cent., and a yellowish-orange with about 1 per cent.

The amount of glucose which is being lost daily may sometimes be required. A twenty-four-hour specimen is then collected and the glucose content estimated accurately. Any of the blood sugar methods in use can be applied to urine, which, however, usually needs diluting in order to bring the concentration of glucose to within the range of the method used. In most cases this means to below about 400 mg. per cent. The dilution necessary can be gauged from the qualitative test. When blood sugar methods are used for urine a small amount of reducing substances is found even in urines which give negative results with Benedict's and Fehling's tests. This corresponds to less than 150 mg. glucose per 100 ml. urine. Estimation before and after the action of yeast will give the amount of fermentable sugar present.

Benedict's quantitative method is commonly used for the estimation of sugar in the urine.

### Determination of Urine Sugar by Benedict's Method

Reduction of an alkaline copper solution is used, but Benedict included potassium thiocyanate and potassium ferrocyanide in his quantitative solution, in order to get a white precipitate and so avoid the formation of a reddish-brown cuprous oxide precipitate. It is then possible to gauge the end-point—the disappearance of the blue colour—much more easily.

**Reagents.** 1. Benedict's Quantitative Solution. Dissolve 200 grams of sodium citrate, 75 grams of anhydrous sodium carbonate and 125 grams of potassium thiocyanate in about 600 ml. of water, with the aid of gentle heating. Filter, cool, and add 18 grams of copper sulphate ($CuSO_4$, $5H_2O$) dissolved in about 100 ml. of water. Pour this in slowly, stirring continuously. Add 5 ml. of 5 per cent. potassium ferrocyanide solution and make up to a litre with distilled water. If the solution is not clear, filter.

2. Sodium carbonate (anhydrous).

**Technique.** Pipette 25 ml. of the Benedict's quantitative solution into a 100 ml. round bottomed flask or beaker and add 3 to 4 grams of sodium carbonate. Introduce a few pieces of porcelain to reduce the risk of bumping. Bring to the boil and, whilst boiling, run in urine slowly from a burette, shaking well at intervals. A white precipitate is formed and the blue colour gradually disappears. Reduce the rate of addition of the urine as the end-point is approached until it is being added drop by drop. The end-point is reached as soon as the blue colour has completely disappeared. The most satisfactory titration is one requiring between 8 and 12 ml. of urine. This corresponds to about 0·5 per cent. of glucose. Use either the qualitative test, or do a quick preliminary titration, to decide what dilution of the urine, if any, is required to give such a final titration.

The final titration should be carried out quickly almost to the end-point, which is then determined carefully by drop by drop addition of the urine. If the flask is allowed to stand after finishing the titration, some re-oxidation may occur because of the action of the air, and a greenish-blue colour then reappears. This should be ignored.

**Calculation.** 0·05 gram of glucose reduces 25 ml. of the alkaline copper solution. Hence

Grams glucose per 100 ml. urine

$$= \frac{0{\cdot}05 \times 100 \times N}{\text{ml. required for the titration}}$$

where N = number of times the urine was diluted.

**Note.** Lactose can be estimated in the same way, but a different factor has to be used in the calculation. 0·067 gram of lactose are required to reduce 25 ml. of the Benedict's solution. Fructose and galactose have a factor 0·053, more nearly the same as glucose.

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*CHAPTER V*

# DIABETES MELLITUS. KETOSIS. DIABETIC COMA

## DIABETES MELLITUS

Ever since the isolation of insulin by Banting and Best in 1921 work has been going on in an endeavour to find out at what point in the metabolism of carbohydrate it acts and to elucidate the pathology of diabetes. For a summary of the present position on these subjects the reader is referred to Himsworth's Oliver Sharpey Lecture (1949) where also a good deal of the relevant literature is cited. For a full discussion of many aspects of these problems see Soskin and Levine (1946).

It now appears that insulin exerts its effect on the reaction in which glucose is converted into glucose-6-phosphate under the influence of adenosine triphosphate, a reaction which, catalysed by hexokinase, forms the first stage both of glycogen formation from glucose, and of the series of reactions by which glucose is oxidized to carbon dioxide and water in the body. It is this reaction which the diabetic person is not able to perform normally. It has been shown by Cori and his co-workers that this hexokinase reaction is inhibited by anterior pituitary extracts, an inhibition which is counteracted by insulin. Adrenocortical extracts also inhibit the reaction. These findings go a good way towards explaining some of the observations on diabetes mellitus made during the years following the introduction of insulin treatment.

It appears that insulin in this way makes possible adequate utilization of glucose by the tissues at normal blood sugar levels. When sufficient insulin is not available, abnormally high blood sugar levels are produced, and this partially restores a more normal rate of glucose utilization. The nature of the increase in blood sugar is discussed by Himsworth in some earlier papers (1939).

Furthermore Himsworth regards diabetes mellitus as a syndrome rather than as a single disease, since it may arise from several causes. Thus it may be due to an actual deficiency in the amount of insulin produced, such as may result from actual destruction of islet tissue in the pancreas. It may also develop when there is increased production of anterior pituitary hormone or adrenocortical hormone. In such cases the amount of insulin produced by the pancreas is not sufficient to inhibit the effect of these hormones. Whatever the cause, glucose utilization is impaired and a diabetic condition is produced.

Himsworth (1939) divided diabetics into two groups, an insulin sensitive type, in which there is actual deficiency of insulin production, and an insulin insensitive group, in which insulin is thought to

be produced normally but is not able to exert its normal effect, possibly because of alterations in other factors referred to above. Insulin sensitivity can be assessed by the insulin-glucose test devised by Himsworth (see p. 54). When this is applied to diabetics, it is claimed by Himsworth that it roughly divides them into these two groups, of which the insulin sensitive are the young diabetics, in whom the disease is severer, and has had a more sudden onset. The patients respond well to insulin, and are more likely both to develop hypoglycæmia from insulin over-dosage and to go into ketosis when treatment is inadequate. On the other hand, there are the insulin insensitive diabetics, who are usually elderly and obese in contrast to the under-weight younger insulin sensitive diabetics. They often have arteriosclerosis, and the disease is relatively mild and insidious in its onset. These diabetics tolerate considerable over-dosage of insulin without going into hypoglycæmia and develop ketosis comparatively rarely.

Recently Lawrence and Bornstein (1951, see also Lawrence, 1951) have studied the amount of insulin present in the circulating blood, and found that, in severe cases with ketosis, insulin was absent, whereas in a series of obese cases with no ketosis, insulin was present in amounts similar to those in normal persons, and that the condition could easily be controlled by low diets without insulin. They used the technique of Bornstein (1950). In this, the plasma under test is injected into alloxan-diabetic rats, which have had their pituitary and adrenal glands removed. The fall in blood sugar is measured, and compared with the result obtained on the same animals previously using crystalline insulin.

Insulin sensitivity appears to decrease with increasing age, and to be reduced by low carbohydrate diets and by injection of pituitary extracts. The diminished sensitivity resulting from low carbohydrate intake has an important bearing on the proportion of carbohydrate which should be included in the diet of diabetics, and has given further support to the tendency to give diabetics a diet containing a higher proportion of carbohydrate than was used formerly.

The characteristic feature of diabetes mellitus then, is a diminished ability to utilize glucose at ordinary blood sugar levels. This produces a diminished glucose tolerance with a raised blood sugar, which is often well above the renal threshold for glucose. Varying degrees of glycosuria result. It is this loss of glucose in the urine which is responsible for several of the most prominent symptoms of diabetes. The osmotic pressure of the glucose present leads to the excretion of an increased volume of urine. So there is a polyuria with frequency of micturition and a pale urine. Up to 5 to 6 litres of urine may be passed daily. The glucose present, however, increases the specific gravity, which may be as high as 1·040. A pale urine with a high specific gravity is characteristic of the condition. A further consequence of the polyuria is the thirst, which is often so marked, and is such a characteristic symptom of diabetes.

To produce the high level of glucose in the blood, the maintenance

of which is made more difficult by the loss of glucose in the urine, increased production of glucose may take place in the liver from amino-acids derived from proteins. If sufficient glucose is not obtained from other sources, body protein—mainly muscle—will be drawn on. This causes the wasting and loss of weight with muscular weakness and lassitude which are found. An increased appetite is often another noticeable symptom, which arises from this heavy loss of material from the body.

There is also increased mobilization of fat. In severer cases more fat is used for supplying the energy requirements of the body. Blood fat and cholesterol are raised (see pp. 194, 188), and there is increased formation of ketone bodies with production of an acidosis, ketosis, which, if the condition is untreated, results eventually in diabetic coma. We shall next consider the tests, qualitative and quantitative, for these ketone bodies.

## KETOSIS

The most important consequence of the increased metabolism of fat in diabetes is the accumulation in the blood of the following ketone bodies :

acetoacetic acid $CH_3.CO.CH_2.COOH$ (diacetic acid).

$\beta$-hydroxybutyric acid $CH_3.CHOH.CH_2.COOH$, formed by reduction of acetoacetic acid.

acetone $CH_3.CO.CH_3$, formed in the body as a secondary product from acetoacetic acid by loss of carbon dioxide.

It is now held that the liver is the main site of formation of the two acids given above. Unless adequate glucose is available, the liver is compelled to use an increased amount of fat, so that these acids are formed in quantities too great for the tissues to metabolize them. They then begin to accumulate in the blood and to be excreted in the urine. It has been shown that diabetics are able to metabolize these substances. It is the increased production which is significant. In addition to the ketosis of diabetes, starvation ketosis arises in the same way. Testing the urine for these substances is an important part of the routine examination of diabetic patients.

### Qualitative Tests for Ketone Bodies in Urine

#### 1. Gerhardt's Ferric Chloride Test

Add 10 per cent. ferric chloride solution, drop by drop, to a few ml. of urine in a test tube. A purplish colour is given by acetoacetic acid. If considerable amounts of phosphate are present a precipitate of ferric phosphate is produced. It may be necessary to filter this off. Add more ferric chloride after filtering and again observe. A similar colour is given by salicylic acid and salicylates. These can be differentiated from acetoacetic acid by their different behaviour towards heat. On thoroughly boiling the urine, acetoacetic acid loses carbon dioxide, and is converted into acetone which no longer

gives the test. If the heating is done after adding the ferric chloride, the colour already formed disappears. Salicylates are unaffected by boiling the urine and the colour persists. This test is only given by acetoacetic acid, not by the other two substances.

### 2. Rothera's Nitroprusside Test

Shake a few ml. of urine with solid ammonium sulphate in excess and add a little sodium nitroprusside, either a few drops of solution or a small quantity of the powdered solid. Add about 1 to 2 ml. of concentrated ammonia. A purple colour is given by acetoacetic acid and acetone. If the ammonia is layered on, a purple ring is obtained at the junction of the liquids. This test is not given by salicylates.

Rothera's test is much more sensitive than Gerhardt's, being positive 1 in 100,000 as compared with 1 in a few thousand for the Gerhardt test.

**Modification using a Powdered Reagent.** Several workers have carried out the test in this form. The following is the technique of Dumm and Shipley (1946). Weigh out 1 gram of sodium nitroprusside (very finely ground), 20 grams of ammonium sulphate, and 20 grams of anhydrous sodium carbonate. Mix completely, but do not grind together. Keep dry. Such a powder will keep for at least a year. Place a small pinch of the powder, 5 mm. in diameter, on a white filter paper. Add 1 drop of urine. Acetone and acetoacetic acid give an immediate violet colour.

The manufacturers of the Clinitest (p. 57) have also produced a tablet form of Rothera's test known as the *Acetest*, in which one drop of urine is placed on a tablet containing sodium nitroprusside, aminoacetic acid, and disodium phosphate. A purple colour is given by acetoacetic acid and acetone.

It will have been noticed that neither of these commonly used tests is given by $\beta$-hydroxybutyric acid, for which there is no test in everyday routine use. However, they almost always occur together. If it is desired to test for this substance, it is necessary to oxidize it to acetoacetic acid. This can be done by means of hydrogen peroxide. Add a few drops of acetic acid to some urine diluted 1 : 1 with distilled water. Boil for a few minutes to remove acetone and acetoacetic acid. Add about 1 ml. of hydrogen peroxide, warm gently, cool, and apply Rothera's test. This is now positive if $\beta$-hydroxybutyric acid was originally present in the urine.

## Determination of Ketone Bodies in Blood and Urine

It is only rarely that biochemists are asked to estimate the amount of ketone bodies present in blood or urine. Dumm and Shipley (1946) have adapted their urine method to give an approximate measure of the acetone and acetoacetic acid present in serum.

### I. Approximate Method of Dumm and Shipley

Dumm and Shipley found that the smallest concentration of acetone and acetoacetic acid which gives a positive test with the

powder described previously is roughly 10 mg. per 100 ml. For blood, carry out the test as for urine but using 1 drop of serum. If this gives a positive test, dilute 1 in 2 and repeat. If still positive, dilute further and continue until no colour is obtained. If, for example, the last specimen to give a positive test was a 1 in 10 dilution, the concentration of acetone and acetoacetic acid is approximately 100 mg. per 100 ml.

### II. Methods of Van Slyke (Van Slyke, 1917, 1929 ; Van Slyke and Fitz, 1917, 1919)

These use the observation of Denigès that when acetone is boiled with mercuric sulphate and sulphuric acid it gives a precipitate many times heavier than the acetone which it contains. Acetone can be estimated either by weighing this precipitate or by titrating the mercury which is present in it. $\beta$-hydroxybutyric acid is first oxidized to acetoacetic acid and this together with any already present is converted into acetone by heating. In this way total acetone bodies can be determined. The amount of the individual substances can be estimated if desired by methods which will be described.

#### Determination in Blood

**Reagents.** 1. Mercuric sulphate, 10 per cent. solution. Dissolve 73 grams of pure red mercuric oxide in 1 litre of 4 N sulphuric acid.

2. Sulphuric acid, 50 per cent. (v/v). Add 500 ml. of acid slowly with mixing and cooling to about 450 ml. of water and then make up to 1 litre with water.

3. Potassium dichromate, 5 per cent. solution.

**Technique.** Proteins are precipitated by means of the 10 per cent. mercuric sulphate solution used for combining with the acetone. Dilute 10 ml. of whole blood with about 100 ml. of water in a 250 ml. volumetric flask, and add 20 ml. of 10 per cent. mercuric sulphate. Shake to coagulate the protein and then dilute to the 250 ml. mark. Stand at least fifteen minutes. Filter. If plasma or serum is used, dilute 8 ml. with 50 ml. of water and 15 ml. mercuric sulphate in a 200 ml. flask.

For the *determination of the total acetone bodies*, measure 125 ml. of filtrate (= 5 ml. of blood or plasma) into a suitable flask. Add 10 ml. 50 per cent. sulphuric acid and 35 ml. of mercuric sulphate. Fit the flask with a reflux condenser and bring to the boil. When boiling has started run in 5 ml. of the 5 per cent. dichromate solution down the condenser tube. It is important that this addition be made after boiling has begun. Then continue to boil gently for an hour and a half. Filter the precipitate *hot*.

It is sufficient in almost all cases to know the total ketone bodies present. The individual ones can be determined. Measure 125 ml. of the protein-free filtrate into a suitable 500 ml. flask and add 10 ml. of 50 per cent. sulphuric acid and 35 ml. of 10 per cent. mercuric sulphate. Boil gently for thirty minutes under a reflux condenser. Filter hot, using a Gooch crucible. This precipitate gives the *acetone*

+ *acetoacetic acid.* Measure 160 ml. of the filtrate (= 4·7 ml. of blood) into a similar 500 ml. flask. Bring to the boil under the reflux condenser, run in 5 ml. of 5 per cent. dichromate down the condenser and proceed as for total acetone bodies. This gives the *β-hydroxybutyric acid.*

For **gravimetric estimation** collect the precipitate in a Gooch crucible, wash with about 200 ml. of cold water, dry for an hour at 110° C., cool and weigh. The precipitate is a mercuric sulphate-dichromate compound of the ketone bodies present.

For **titrimetric estimation** the procedure is as follows.

**Reagents.** 1. N. hydrochloric acid.

2. Sodium acetate, 3 M solution. 408 grams of $NaC_2H_3O_2, 3H_2O$ per litre of solution.

3. Mercuric chloride, 0·05 M solution. Dissolve 13·58 grams of mercuric chloride in water and make up to 1 litre. To standardize, dilute 25 ml. to about 100 ml., pass hydrogen sulphide until the black precipitate flocculates and leaves a clear solution. Collect the mercuric sulphide on a Gooch crucible, dry at 110° C. and weigh. 0·2908 grams of precipitate should be obtained.

4. Potassium iodide, 0·2 M solution. Dissolve 36 grams of potassium iodide in water and make up to a litre. Measure 20 ml. into a flask and titrate with the mercuric chloride until a permanent red precipitate appears. Rather more than 20 ml. will be needed. Add the necessary amount of water to the iodide so that 20 ml. of the chloride titrates 20 ml. of the iodide. Thus 1 ml. of 0·05 M. $HgCl_2$ = 1 ml. 0·2 M. KI according to the equation

$$HgCl_2 + 4\ KI = K_2HgI_4 + 2\ KCl.$$

**Technique.** Instead of using a Gooch crucible, filter hot through an ordinary filter paper, wash as above, transfer completely to a small beaker and break up with a rod in 15 ml. of normal hydrochloric acid and heat to dissolve the precipitate. For a good end-point in the titration the acidity of the solution must be lessened. To do this add 6 to 7 ml. of 3 M. sodium acetate to the cooled solution after the precipitate has been redissolved. Then run in 0·2 M potassium iodide rapidly with constant stirring. A red precipitate of mercuric iodide forms immediately if more than a small amount of mercury is present, redissolving as soon as 2 or 3 ml. of potassium iodide in excess of the amount needed to form the soluble $K_2HgI_4$ have been added. If only a few mg. of mercury are present, an excess of potassium iodide may have been added before the mercuric iodide could precipitate, so the solution remains clear. If this occurs, add at least 5 ml. of the 0·2 M iodide. Note the volume of iodide added and titrate the excess with 0·05 M mercuric chloride until a permanent red precipitate is obtained.

**Calculation.** All results are expressed as mg. acetone per 100 ml. of blood. They are calculated as follows :—

| | | |
|---|---|---|
| Gravimetric : | total acetone bodies | = 1·24 × mg. precipitate. |
| | acetone + acetoacetic acid | = 1·00 × mg. precipitate. |
| | β-hydroxybutyric acid | = 1·40 × mg. precipitate. |

Titrimetric : total acetone bodies = 16·1 × ml. 0·2 M KI.
acetone + acetoacetic acid = 13·0 × ml. 0·2 M KI.
β-hydroxybutyric acid = 17·2 × ml. 0·2 M KI.

The ml. 0·2 M KI in this calculation are obtained by subtracting the ml. 0·05 M $HgCl_2$ used in the back titration from the ml. 0·2 M KI added previously.

### Determination in Urine

**Additional Reagents.** 1. Copper sulphate, 20 per cent. solution of $CuSO_4$, $5H_2O$.

2. Calcium hydroxide, 10 per cent. suspension. Mix 100 grams of calcium hydroxide with a litre of water.

**Technique.** It is necessary to remove glucose and other interfering substances. Pipette 25 ml. of urine into a 250 ml. measuring flask. Add 100 ml. of water and 50 ml. of the copper sulphate solution. Mix, add 50 ml. of 10 per cent. calcium hydroxide suspension and mix again. If not alkaline to litmus add more calcium hydroxide. Make up to the mark, stand half an hour and filter. This will remove up to 8 per cent. of glucose. It should be carried out even when glucose is absent since it is needed to remove other interfering substances. The filtrate can be tested for completeness of removal by boiling a few ml. in a test tube. Glucose, if still present, gives a precipitate of cuprous oxide.

For *total acetone bodies*, pipette 25 ml. of the urine filtrate into a 500 ml. flask. Add 100 ml. of water, 10 ml. of 50 per cent. sulphuric acid and 35 ml. of 10 per cent. mercuric sulphate. Bring to the boil under a reflux condenser and after boiling has begun run 5 ml. of 5 per cent. dichromate down the condenser tube. Boil gently for one and a half hours and complete as described above for blood.

For *acetone and acetoacetic acid*, boil 25 ml. urine filtrate, 100 ml. of water, 10 ml. 50 per cent. sulphuric acid and 35 ml. mercuric sulphate in a 500 ml. flask under a reflux gently for thirty minutes. Filter and complete the estimation as before.

For *β-hydroxybutyric acid*, add 100 ml. of water and 2 ml. of 50 per cent. sulphuric acid to 25 ml. urine filtrate and boil in an open flask for ten minutes. This removes acetoacetic acid and acetone. Add enough water to make up the original volume of 127 ml., add a further 8 ml. of 50 per cent. sulphuric acid and 35 ml. 10 per cent. mercuric sulphate. Bring to the boil under the reflux, add the dichromate and proceed as before.

**Calculation.** All results are as grams acetone per litre of urine.

| | |
|---|---|
| acetone + acetoacetic acid . | 20·0 × grams precipitate.<br>0·26 × ml. 0·2 M KI. |
| β-hydroxybutyric acid . . | 26·4 × grams precipitate.<br>0·344 × ml. 0·2 M KI. |
| total acetone bodies . . | 24·8 × grams precipitate.<br>0·322 × ml. 0·2 M KI. |

**Reagent Blank.** No precipitate whatever should be produced when the complete estimation of total acetone bodies, including the

preliminary copper sulphate treatment, is carried out with distilled water instead of urine. This should always be done.

**Blank Determination of Precipitate from other Substances in Urine.** Treat 25 ml. of urine filtrate with sulphuric acid and water as above and boil to drive off acetone. Make up the volume to 175 ml. with the same amounts of sulphuric acid and mercuric sulphate used above. Reflux for forty-five minutes without dichromate. The precipitate obtained should be determined and subtracted from the values obtained in the above estimation.

**Other Method.** Behre and Benedict (1926) and Behre (1928, 1940) developed a method in which the ketone bodies are converted into acetone. This then reacts with salicyl aldehyde in the presence of alkali. The amount of red-coloured di(hydroxybenzal)-acetone thus formed is measured colorimetrically.

INTERPRETATION

Normal blood contains up to 3 mg. per 100 ml. In diabetes as much as 300 to 400 mg. per 100 ml. and over has been observed. In normal urine up to 50 mg. of acetone bodies (as acetone) may be excreted daily whilst in diabetes 10 to 50 grams per litre may be found, the greater part being usually $\beta$-hydroxybutyric acid.

## TESTS IN THE TREATMENT OF DIABETES AND IN DIABETIC COMA

In the ordinary treatment of diabetes it is usual to rely on urine tests for sugar and ketone bodies, sometimes with, and sometimes without, periodic estimations of blood sugar. In the stabilizing period in hospital daily blood sugars make possible quicker adjustments of diet and insulin. It has also to be remembered that urine sugar tests can be rather misleading, since the renal threshold may vary appreciably. At best they only tell us that at a particular time the blood sugar was above or below a value somewhere in the region of 200 mg. per 100 ml. Furthermore, a particular urine specimen may be formed over a long period of time during which there can be considerable change in blood sugar. Short-term urine specimens are much more valuable. Although many physicians do rely solely on urine tests, there would appear to be little doubt that as Lawrence states (1950), treatment is safer and easier if blood sugar determinations are done. They can now be carried out so simply and quickly that there is little reason why they should not be done in the required number by any well organized laboratory.

If blood sugar estimations are carried out it is important to note the time, so that they can be correlated with food and insulin. The course of the blood sugar varies considerably according to the type of insulin which is being given. If a single daily blood sugar is done on a patient in hospital, the time most commonly chosen is the late morning. If ordinary soluble insulin is being given, the course of the

blood sugar in a reasonably well-controlled case, is as follows. Before breakfast it is usually somewhat raised, perhaps to just over the threshold level. The effect of the morning insulin, given about half an hour before breakfast, is to bring about a progressive fall in blood sugar during the morning, so that it may well be within normal fasting limits about noon. After the midday meal, before which no insulin will have been given, the blood sugar tends to rise, and may reach and even slightly exceed the threshold in the late afternoon. After an evening meal, again preceded by insulin, we get a progressive fall similar to that in the morning back towards normal limits, which may be reached in the late evening. After this a rise tends to occur, though rather more slowly than in the afternoon, since food will not have been taken. As a result the blood sugar will again be appreciably above normal by the time the morning insulin is to be taken. Thus a single late morning blood sugar done shortly before the midday meal may safely be within normal fasting limits.

On the other hand, if protamine zinc insulin is being used, because of the delayed action of this insulin, which in large doses acts up to twenty-four hours after its injection, the blood sugar follows a different course. With a really satisfactory dose of this alone, the blood sugar may be within normal fasting limits shortly before breakfast when the insulin is given. The effect of breakfast is to bring about a steady rise in blood sugar up towards the threshold level in the next two or three hours so that the blood sugar in the late morning may be in the region of 200 mg. per 100 ml. During the afternoon the insulin begins to make itself felt and the blood sugar tends to fall nearer to normal only to be sent up again by the evening meal, after which, during the late evening, it begins to fall again and is in the region of normal fasting limits just before breakfast when the next dose of insulin is given. So with this type of insulin late morning blood sugars should be appreciably higher than with ordinary soluble insulin. Fasting blood sugars taken before breakfast may be a better guide since they should give an indication as to how low the blood sugar is going, but these are not so convenient to arrange as are late morning or late afternoon estimations.

Mixtures of the two insulins are sometimes used as one daily dose given in the morning before breakfast. In this case the late morning sugar is nearer to normal limits, as is the fasting blood sugar. A rise occurs in the afternoon and early evening after which the delayed action of the protamine zinc insulin acts to bring down the blood sugar towards normal by the following morning.

Globin insulin has also been used. It has an action intermediate between the protamine zinc and the soluble insulins. For fuller accounts of the action of the different insulins and of their use in treatment, see Lawrence (1950).

In addition to the blood sugar estimation, the urine is tested for the presence of glucose. It will be seen that a small amount of glucose in the early morning urine may be expected when soluble insulin is being used whilst the late morning specimen may be sugar free. In no urine specimen, however, should much sugar be found.

Qualitative tests for ketone bodies should be carried out at intervals, particularly when the glycosuria becomes more marked and when the blood sugar level is appreciably raised. Their presence is an indication that a more serious stage of the disease has been reached. Their presence or absence is a better indication of the severity of the condition than is the amount of sugar in the urine.

The chemical findings in diabetic (hyperglycæmic) coma and in hypoglycæmic coma will now be discussed.

**Diabetic (Hyperglycæmic) Coma.** As already stated, it is the accumulation of the keto-acids, due to their formation at a rate greater than that at which the body is able to remove them, that is responsible for the production of the body changes which lead to the development of the coma.

The *urine* contains sugar and ketone bodies in large amounts. It often also contains a small to moderate quantity of albumin and some granular casts may be seen in the urine deposit.

The *blood sugar* is usually found to be between 400 and 1,000 mg. per 100 ml. but higher values may be obtained occasionally. The author has seen several between 1,000 and 1,400 and one of 2,400 mg. per 100 ml., whilst Lawrence has recorded one finding of 2,060 mg. per 100 ml. Frequent estimations of blood sugar during the comatose stage give a valuable guide to treatment. The most satisfactory level of blood sugar to be aimed at so long as coma is present is a subject of contention. It may perhaps best be put in the region of 300 mg. per 100 ml. at which an increased rate of glucose utilization is obtained without producing too much glycosuria with its ill-effects on water and electrolyte distribution.

The accumulated keto-acids can be removed up to a point by being excreted in the urine, thus giving a highly acid urine, and the kidneys may also be able to excrete some as the ammonium salts. In the severer cases, however, their removal involves loss of fixed base. The plasma bicarbonate has to be used for their neutralization so that they are then excreted as the sodium salts. The result is a decrease in plasma bicarbonate, that is, in the *alkali reserve*. Estimation of this (see p. 354) is often carried out in diabetic coma and precoma. The alkali reserve, expressed as ml. of carbon dioxide which can be liberated from the bicarbonate present in 100 ml. of plasma, falls progressively as the condition becomes worse, from the normal range of 53 to 75 volumes per cent., with the production of an increasing degree of acidosis, until at below 15 volumes carbon dioxide per cent. coma supervenes.

A further result of this excretion of base is to help to produce a state of dehydration. The loss of electrolytes and accompanying fluid, together with the effect of the polyuria caused by the excretion of the glucose, and the vomiting which quite frequently occurs in diabetic coma, all contribute to this end. There is consequently, often a low plasma sodium and chloride partly masked by the hæmo-concentration. The reduction in plasma volume ultimately produces a circulatory failure, with low blood pressure. This, by affecting the renal blood flow, is probably responsible for the *increased*

*blood urea*, which is often found in diabetic coma. Increased breakdown of protein may also be a contributory factor. The blood urea is frequently found to be between 40 and 150 mg. per 100 ml. Much higher values are found if there is complicating antecedent renal failure as in cases with the Kimmelsteil-Wilson syndrome. Clinically the dehydration is shown by the dry skin.

The typical breathing found in diabetic coma—Kussmaul breathing—has been attributed to the effect of the enol form of the acetoacetic acid on the respiratory centre.

The onset is relatively insidious, usually a matter of a few days. The effect of infection as a precipitating agent is important. Treatment aims at correcting the above abnormalities. Adequate insulin by increasing glucose utilization stops the excessive formation of ketone bodies, whilst intravenous saline may be used to remove the dehydration responsible for the circulatory failure which is the cause of many of the deaths in diabetic coma. For full accounts of treatment, see the books of Lawrence (1950) and Joslin *et. al.* (1952).

During treatment profound changes in certain blood constituents may develop as a result of the large amount of carbohydrate being metabolized. Thus plasma inorganic phosphorus may fall precipitously to below 1·0 mg. per 100 ml. and plasma potassium to the region of 10 mg. per 100 ml. with flaccidity of the muscles arising from the latter of these changes. There is also a considerable fall in plasma magnesium. These changes may have an adverse effect on the patient's condition. A marked increase in blood pyruvic acid has also been reported during the treatment of diabetic coma, due presumably to a deficiency of cocarboxylase resulting from a shortage of phosphate for the phosphorylation of vitamin $B_1$.

For a recent discussion of laboratory investigations in the control of diabetic coma, see Gray (1951).

**Hypoglycæmic Coma.** This condition arises most commonly from an over-dosage of insulin, but may be due to insulin-secreting islet tumours in the pancreas, and may occur in Simmonds' disease and Addison's disease. The *blood sugar* is usually below 40 mg. per 100 ml., although the level at which symptoms of hypoglycæmia begin to appear, depends to some extent on the level of blood sugar to which the patient has been accustomed. A diabetic patient, who has had a consistently raised blood sugar, may show symptoms when the blood sugar is markedly reduced, even though the blood sugar is still appreciably higher than that usually found in hypoglycæmic coma.

The urine contains no sugar, ketone bodies, or albumin unless the blood sugar has been rapidly brought down from very much higher levels, as is sometimes the case in the treatment of diabetic coma.

There is no acidosis, so that the alkali reserve is normal. There is also no dehydration, and instead of the circulatory failure with low blood pressure, beads of perspiration may be seen as a contrast to the dry skin of hyperglycæmic coma. The blood urea is normal. There is no vomiting.

The onset is usually sudden, a matter of minutes, and treatment

with carbohydrate is rapidly effective in most cases. A blood sugar to confirm the diagnosis is all that is required from the laboratory.

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## *CHAPTER VI*

# PROTEINS IN URINE. ALBUMINURIA

Proteins found in urine in various pathological conditions include the plasma proteins, plasma albumin, globulin and fibrinogen; hæmoglobin from the red blood cells, and methæmoglobin derived from it; abnormal proteins, such as Bence-Jones' protein and proteoses, filtered from the plasma; added proteins derived from pus and mucus from the urinary tract, and from semen and vaginal secretions. These give positive results with the commonly used routine tests for protein in urine. Although the term proteinuria is the more accurate one, albuminuria is still widely used in this more general sense. We shall consider first the routine tests in common use for detecting protein in urine.

### Tests for Protein in Urine

1. **The Boiling Test.** *Reagent:* Acetic acid, 33 per cent. (v/v).

**Technique.** Make the urine slightly acid to litmus by adding the dilute acetic acid drop by drop. Filter if the urine is not quite clear. It is not necessary to filter very slightly turbid urines if appreciable amounts of protein are present. Bacteria are not removed by filtering, but are by shaking the urine with a little kieselguhr, and then filtering.

Fill a test tube about three-quarters full with the urine and heat the top centimetre or two to boiling. Protein present is coagulated and can be seen by comparing with the unboiled urine lower down the tube. Comparison against a dark background is useful in detecting traces of protein.

It is important that the *p*H should be on the acid side of neutral. With alkaline urines proteins may not be precipitated owing to the formation of alkaline metaprotein which is not coagulated. Quite a large amount of protein may be present and not be detected in such circumstances. Furthermore, on heating alkaline urines phosphates may be precipitated (see p. 308).

It is a common practice to heat the clear urine before making acid with the acetic acid. A precipitate may then be due either to protein or phosphates, or both. Acetic acid is then added and dissolves any phosphates present. Even if no precipitate appears it is advisable to acidify since sometimes an alkaline urine which has not given a precipitate on heating will give one when made acid. Whilst for the most part this gives similar results to those obtained by first making acid to litmus and then boiling, false results may be obtained occasionally.

Purdy adds to the urine one-third its volume of saturated sodium chloride solution, and then 5 to 10 drops of 50 per cent. acetic acid

to a test tube about two-thirds full. After mixing, the top inch is heated. Precipitation of mucus is prevented by the salt present.

2. **Salicylsulphonic Acid Test.** To 5 ml. of clear urine add 0·5 ml. of 25 per cent. salicylsulphonic acid. Protein appears as a white precipitate. Compare with untreated urine if only traces are present.

3. **The Nitric Acid Test.** Layer urine carefully on to a few ml. of concentrated nitric acid in a test tube so as to get a sharp line of demarcation. Proteins give a white ring at the junction of the fluids.

Both salicylsulphonic acid and nitric acid tests give false positives with uroselectan used for X-rays of the urinary tract, and with uric acid in concentrated urines. Urea in high concentration can give a precipitate with nitric acid. If such concentrated urines are diluted, false positives are no longer obtained.

The boiling test and the salicylsulphonic acid tests are the ones most commonly used. Properly carried out the former is perhaps the best single test. The very small amount of protein which may be present in normal urines is not sufficient to give a positive result with these tests.

The salicylsulphonic acid and nitric acid tests are positive with all the proteins mentioned above. As will be seen later, Bence-Jones' protein may be missed with the heating test.

## INDIVIDUAL PROTEINS AND THEIR DETECTION

### 1. Plasma Proteins

**Albumin and Globulin.** The proteins most commonly found in the urine are those derived from the blood plasma and consist of a mixture of plasma albumin and globulin. These are not normally filtered through the glomeruli of the kidneys, but in disease the glomerular permeability may be altered so that the passage of some albumin and a smaller amount of globulin becomes possible. The albumin of blood plasma has a smaller molecular weight than the globulins and so is more easily lost, particularly with smaller changes in permeability.

Albumin is only precipitated by completely saturating the urine with ammonium sulphate, whereas half saturation is sufficient to precipitate the globulins. Mix equal volumes of urine and saturated ammonium sulphate solution. The albumin present remains in solution and can be separated from the precipitated globulins by filtering. Globulins are also precipitated by complete saturation with magnesium sulphate. Of the globulin fractions using the Howe fractionation (see p. 161), *pseudoglobulin* fractions are soluble in water, whilst *euglobulin* fractions are not. In regard to the euglobulin see also p. 87.

**Fibrinogen,** having a larger molecular weight than the globulins, tends to be found in the urine only when a good deal of blood or plasma has been added at some point. In such cases fibrin clots may sometimes form.

The significance of the occurrence of these plasma proteins will be considered later (see p. 87).

### 2. Blood in Urine

Sometimes, as in acute glomerulonephritis, the glomeruli may be so damaged that blood is able to pass directly into the tubules and hence out in the urine. Alternatively, in patients with lesions of the urinary tract, blood may be introduced after the urine has left the tubules. In such cases we have the condition known as *hæmaturia*, in which red blood cells are present in the urine, at least in a fresh specimen. Hæmaturia is often visible to naked eye inspection, but slighter degrees may only be revealed by microscopic examination.

In any condition in which there is intravascular hæmolysis of red blood cells with liberation of hæmoglobin into the plasma, above a certain low plasma level this will be filtered at the glomeruli and appear in the urine. Red blood cells will not be seen. To this finding the term *hæmoglobinuria* is given. In hæmaturia, hæmoglobin may be liberated as a result of hæmolysis after the blood has been mixed with the urine, and this will increase in extent on standing. So in hæmaturia we have red cells present, probably with some hæmoglobin, whilst in hæmoglobinuria there is hæmoglobin without any red cells. Hæmoglobin may be converted to methæmoglobin in the cells. If these are hæmolysed methæmoglobin will be liberated and will be excreted in the urine. The same change may occur to hæmoglobin in urine which is allowed to stand.

**Tests for the Presence of Blood.** These include microscopic examination for red blood cells, spectroscopic examination for blood pigments, and chemical tests for blood. The most sensitive test so long as red cells are present is microscopic examination. Red cells may be seen microscopically when both other tests are negative. See p. 99 for presence of red cells in the urine deposit, p. 394 for spectroscopy, and p. 216 for chemical tests.

### 3. Abnormal Proteins from Plasma. Bence-Jones' Protein

In some diseases abnormal proteins may be present in plasma and be filtered at the glomeruli. Some proteoses belong to this group, but the most important and interesting member is the protein known as Bence-Jones' protein, which, when found, is almost always associated with multiple myeloma of bone. Recognition of its presence in the urine may thus be an important diagnostic aid in this disease. It is probably a group of rather similar proteins, in some respects resembling the globulins, with which it is estimated on ordinary fractionation. The most characteristic property of Bence-Jones' protein is its behaviour on heating. Whereas ordinary plasma albumin and globulin begin to precipitate between 60° and 70° C., Bence-Jones' protein begins to come down between 40° and 60° C., precipitates completely at a relatively low temperature, and then redissolves as the temperature reaches boiling point. Subsequent cooling reprecipitates the protein and boiling redissolves it.

### Tests for Bence-Jones' Protein

1. **Bradshaw's Test.** Layer a few ml. of urine carefully on to a few ml. of eoneentrated hydroehlorie aeid in a test tube so as to obtain a good line of demarcation. Bence-Jones' protein is precipitated by the acid and gives a white ring at the junction of the two fluids. If the test is negative, Bence-Jones' protein may be considered absent, but a positive test does not neeessarily indieate its presence. The heating test should be used to confirm. Slightly positive tests may be obtained with Bradshaw's test if other proteins such as albumin are present in eonsiderable amounts. With Bence-Jones' protein the test will still be positive after appreeiable dilution of the urine.

2. **The Heating Test.** The coagulation of Bence-Jones' protein is much influenced by the *p*H of the urine and by the concentration of salts present in it. As suggested by Harrison (1947) it is best to put up three tubes of different aeidity. Filter the urine if it is not elear, and adjust to a *p*H just faintly acid to litmus. Put a few ml. of this urine into each of three test tubes. To the seeond and third add respectively 1 and 2 drops of 33 per cent. acetie acid. Place the tubes into a water bath and raise the temperature gradually. It is better to plaee the thermometer inside the tubes in the urine itself. Observe the tubes earefully for the first signs of any precipitation of protein. Benee-Jones' protein begins to come down between 40° and 60° C., often in the forties, whilst albumin and globulin do not do so until the temperature is above 60° C. Continue heating up to the boiling point. Benee-Jones' protein then redissolves. It may be neeessary to remove from the water bath and boil over a flame.

In some eases albumin is present in addition to the Bence-Jones' protein. They should be separated by filtering hot, through a hot filter funnel. The Benee-Jones' protein comes through leaving the albumin behind on the filter paper. On cooling the filtrate the Benee-Jones' protein repreeipitates and can then be eompletely redissolved on once again heating to boiling point. When there is a mixture of albumin and Benee-Jones' protein, reliance should not be placed on an apparent deerease in the amount of the precipitate on boiling the urine.

Cook (1951) adds sufficient 25 per cent. salieylsulphonic acid to precipitate all the protein and then boils gently over a Bunsen burner. Benee-Jones' protein dissolves so that its presence is shown by a lessening or disappearance of the turbidity whieh returns on cooling. When other proteins are also present the boiling mixture is filtered through a heated funnel, using Whatman paper No. 541. Bence-Jones' protein precipitates when the filtrate is cooled. The final filtrate must be acid.

A further test which can be used is :

3. **Osgood and Haskin's Test.** Add 0·5 ml. of 50 per cent. acetic acid and 1·5 ml. of saturated sodium chloride solution to 2·5 ml. of urine. A precipitate at ordinary temperatures appearing on addition of the salt solution is strongly in favour of the presence of Bence-

Jones' protein. Heat the mixture gradually, noting the temperature at which any change occurs. If Bence-Jones' protein is present the precipitate increases in amount at 40° C. to redissolve on boiling. Albumin and globulin usually appear only on heating, and boiling has no effect on the amount of the precipitate.

### 4. Mucus

In addition to the proteins already considered, mucus may be present. Normal urines may contain a small amount, which may form a faint cloud on standing. Mucus occurs in greater amounts in inflammatory conditions of the urinary tract, as a result of prostatic massage, and when the urine contains semen or vaginal discharge.

**Test for Mucus.** Mucus is distinguished by being precipitated by acetic acid in the cold. One of the globulin fractions which may be present in urine, the *euglobulin*, or as it is sometimes known, *lipoid globulin*, is also precipitated, but dissolves in excess of acetic acid whereas mucus does not. It is advisable to dilute concentrated urines. A useful procedure is as follows. Dilute a few ml. of urine with an equal volume of water. To this add 33 per cent. acetic acid, drop by drop, comparing with untreated urine if necessary. Mucus and, or, euglobulin gives a precipitate or opalescence. Add further acetic acid. If the precipitate is due to mucus it reaches a maximum and remains unchanged, if due to euglobulin it redissolves in the excess of acetic acid.

### Classification of Albuminuria. Interpretation

The most useful classification is the one which divides cases of albuminuria into two main groups, organic and functional according to whether the albuminuria can or cannot be shown to be associated with any organic disease. Organic albuminuria is then further subdivided into three groups according as the condition is primarily renal: pre-renal, when the kidneys are only affected secondarily to some other disease: or post-renal, when the protein, in this case often not plasma protein, is added to the urine after it has left the renal tubules.

**Functional Albuminuria.** The albuminuria in such cases is usually small in amount, intermittent, and is accompanied by no other symptoms or evidence of renal disease. Urine deposits are normal, as are renal function tests. This type of albuminuria is often discovered on routine medical examination. It is most frequently found in young people in their teens. The albuminuria may be connected with posture, being absent when the person is lying down and present when standing. This type of albuminuria usually clears up in early adult life, and people with it do not appear to be more likely to develop renal disease subsequently than do other young people of their age. The differentiation from cases having some renal defect is not always easy, but a general working rule is to regard as harmless an albuminuria, slight in amount, in a young person who has no other signs suggesting renal disease and no previous history of this condition.

It should be noted that any severe stress may lead to a transient albuminuria. Exeessive physieal aetivity and exposure to severe eold are examples.

A small amount of albumin may be found in the urine in a few per eent. of eases during the last few weeks of pregnaney. This is also functional being aeeompanied by no other sign of renal impairment. Sueh albumin usually disappears within a few days after delivery. It is probably due to some renal eongestion resulting from the pressure of the fœtus affeeting the renal eireulation. The albuminuria aeeompanying an attaek of eelampsia is usually mueh heavier and there are other urine abnormalities and clinieal signs.

The protein in these eases of funetional albuminuria is plasma albumin with a little globulin.

**Organic Albuminuria.** 1. **Pre-renal Albuminuria.** Albuminuria is found in a wide range of eonditions whieh produee it beeause of seeondary effeets on the kidneys. The albuminuria usually disappears when the primary disease is eured. In most eases the albuminuria is due to some impairment of the renal eireulation. Thus we find some degree of albuminuria when there is dehydration, for example, as a result of the vomiting in pylorie obstruction, in intestinal obstruetion, in severe diarrhœas, in diabetie eoma, in Addison's disease ; in heart disease with passive eongestion of the kidneys ; when there is intra-abdominal pressure as in aseites and intra-abdominal tumours ; in fevers, possibly due to toxie effeets ; in some blood diseases, especially severe anæmias, possibly due to nutritional factors ; whilst some drugs may have a toxie effeet on the kidneys. The dividing line is not always sharp from renal albuminuria sinee in some eases changes in the kidneys may oeeur. With improvement in the primary disease, however, the kidney changes usually disappear. Again the protein is a mixture of plasma albumin and globulin.

2. **Renal Albuminuria.** Albuminuria is naturally found in all forms of renal disease, whether inflammatory, degenerative or destruetive. Whilst the proteins are usually plasma albumin and globulin, red blood eells and hence blood pigments may sometimes be present.

3. **Post-renal Albuminuria.** As we have seen protein may be added to the urine as it passes along the urinary traet. Lesions of the renal pelvis, bladder, prostate and urethra ean all lead to such an addition. Seeretions and discharges from the vagina and admixture of semen may also add protein and give positive tests. Urines eontaining pus will also usually give positive tests. Blood also may be added at this stage. The type of protein is obviously mueh more variable than in the other types of albuminuria. The eause of the positive tests for protein in these eases ean often be reeognized by means of a mieroseopie examination of the urine deposit. The term *false albuminuria* has been applied to this group sinee the positive test may wrongly lead to a suspicion that renal disease is present.

## Quantitative Estimation of Protein in Urine

The estimation of the amount of protein lost in the urine is not as a rule important apart from the rough indication which can be obtained from the qualitative tests. However, it may sometimes be of value to know the amount of protein which is being lost in the urine daily. Several methods are in use, varying considerably in the degree of accuracy. Esbach's method, one of the simplest, is convenient and is used comparatively frequently, though it is only approximate.

### I. Esbach's Method

**Esbach's Reagent.** Dissolve 5 grams of picric acid and 10 grams of citric acid in distilled water and make up to 500 ml.

Fig. 20. Esbach's Albuminometer.

An Esbach's Albuminometer is used (see Fig. 20). Add urine to the mark U, followed by reagent to the mark R. Stopper, and mix by inversion several times. Allow to stand twenty-four hours. Then read off the amount of protein directly on the tube, which is calibrated in parts per 1,000, that is grams of protein per litre of urine. If the urine is a concentrated one dilute it with water to a specific gravity of about 1·008–1·010, and make the necessary correction to the reading.

Other approximate methods involving centrifuging in specially calibrated tubes, such as Aufrecht's tube, are not recommended, but the turbidimetric method, also an approximate one, described for estimating cerebrospinal fluid protein (see p. 462), can be used for determining urine protein content.

### II. Turbidimetric Method (King and Haslewood, 1936)

The preparation of the standard tubes is described under cerebro-spinal fluid protein (p. 462). The technique is similar. To 1 ml. of urine in a small test tube similar to those used for the standards, add 3 ml. of 3 per cent. sulphosalicylic acid and after standing for five minutes compare with the standards. Most urines require diluting so as to bring the protein within the 0–100 mg. per 100 ml. range. As stated (p. 463) this method can be adapted for use with the photoelectric colorimeter if standards are not available. For the blank use 1 ml. of urine or diluted urine plus 3 ml. of water.

The above methods are sufficiently accurate for almost all ordinary purposes. For a more accurate determination the Kjeldahl method or one of the colorimetric methods given for plasma proteins may be

used. The biuret method has been adapted to urine by Hiller, McIntosh and Van Slyke (1927).

INTERPRETATION

The greatest amounts of protein in the urine are found in nephrosis, or using Ellis's classification (Ellis, 1942) in the early stage of Type II nephritis. There may then be several per cent. present and as much as 20 to 50 grams of protein may be lost daily. In fact losses of up to 100 grams a day have been reported. The protein is largely albumin, the ratio of albumin to globulin being more than 10 : 1. Smaller amounts are found in the other renal conditions and there is a greater proportion of globulin. As a rule there is more protein in the urine in acute glomerulonephritis (Ellis, Type I) than in chronic glomerulonephritis (corresponding to the later stages of Type II nephritis of Ellis). In the former of these a common finding is between 0·5 and 1·0 per cent. with a wider range of from 0·2 up to and occasionally over 2·0 per cent. The ratio of albumin to globulin is usually between 10 : 1 and 5 : 1. In chronic glomerulonephritis it is the rule to find less than 0·5 per cent. of protein in the urine and with impending uræmia only traces of protein are present in some cases. The ratio of albumin to globulin may be less than 5 : 1.

In functional albuminuria the majority of cases show less than 0·2 per cent. although a little more may be found occasionally.

A variable amount of protein is found in the urine in the various destructive lesions of the kidneys but this never approaches in quantity that found in nephrosis.

While the amount of Bence-Jones' protein which may be exereted daily varies greatly, it may reach 50 grams and even on occasion be a little higher than this.

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*CHAPTER VII*

# URINARY DEPOSITS

In assessing the significance of albuminuria, further laboratory investigations will usually be carried out. Examination of any urinary deposit will sometimes yield valuable information either of a positive or negative character. The many constituents of such deposits can be classified into three main groups :

(*a*) Chemical substances, crystalline or amorphous.
(*b*) Cells, from the blood or the various parts of the renal tract.
(*c*) Casts.

The last two groups have been described as organized, the chemical substances as unorganized constituents. In addition a number of objects may be found in urine deposits, mostly extraneous, such as hairs. We shall consider these groups in turn.

## CHEMICAL SUBSTANCES

These include substances present both physiologically and pathologically, and may be listed as follows :

Phosphates—ammonium magnesium phosphate, calcium hydrogen phosphate, magnesium phosphate.
Calcium oxalate.
Calcium carbonate.
Calcium sulphate.
Uric acid.
Urates—ammonium, sodium, potassium, calcium and magnesium.
Amino acids—cystine, tyrosine, leucine.
Organic substances such as hippuric acid, cholesterol, indigo, xanthine, bilirubin, and the sulphonamide drugs.

Of these, phosphates, oxalates, uric acid and urates are by far the most commonly seen.

Important general points to note are the *p*H of the urine from which the deposit was obtained, the behaviour of the deposit when the urine is warmed, and the substances in which the deposit is soluble. Thus phosphates are deposited in alkaline urines (or occasionally in slightly acid urines (see below) ) as is ammonium urate. Uric acid and metal urates are seen in the deposit from acid urines, whilst calcium oxalate may be found in all urines. Uric acid and urates are deposited when the urine cools and redissolve on warming. Phosphates dissolve in dilute acetic acid, oxalates in dilute mineral acids, uric acid in neither. Other solubilities will be discussed as the various substances are considered. Finally the colour of the deposit should also be noted. Uric acid and urate

deposits are often coloured, whilst if red blood cells are present these will also give a coloured deposit.

## Phosphates

Crystals of **ammonium magnesium phosphate**—" triple phosphate " —are found in alkaline urines, most typically when bacterial action has occurred with production of ammonia. This may occur either internally, when there is infection of the urinary tract, or externally when the urine has become contaminated with bacteria after being passed. Examples of these crystals are shown in Fig. 21. They are colourless and tend to be of two types, either prisms with three, four,

Fig. 21. Triple Phosphate Crystals.

or six sides and oblique surfaces at the ends, the so-called coffin lid type, or the feathery, fernlike variety. The former are much more commonly seen in urine deposits. Triple phosphate crystals are often seen in conditions in which there is retention of urine, particularly if there is some infection of the urinary tract as well.

**Calcium hydrogen phosphate,** though most often found crystalline in the form of rosettes or star-shaped clusters—" stellar phosphates " —as shown in Fig. 22a, also occurs in irregular colourless plates or flakes (Fig. 22b), which may be derived from a pellicle which forms on the urine after standing. Stellar phosphates occur less frequently than triple phosphates. In addition to occurring in alkaline urine, they may be found in acid urines with a *p*H between 6 and 7.

**Magnesium phosphate** is found as long rhombic plates in alkaline to weakly acid urines. It is much rarer than triple or stellar phosphates.

**Amorphous Phosphates.** Earthy phosphates can also give an amorphous deposit in the form of fine granules or small spheres. These are colourless and so differ from amorphous urates which are usually coloured (see p. 95). They can also be distinguished by

(a) As stellar phosphates.

(b) As plates.

FIG. 22. Calcium Hydrogen Phosphate Crystals.

differences in solubility and in the *p*H of the urine from which the deposit was obtained.

Deposits of phosphates dissolve in dilute acetic acid (33 per cent.).

## Uric Acid

Crystals of uric acid occur quite frequently in deposits from acid urines. Whilst pure uric acid crystals are colourless, those found in urine deposits are almost always coloured. This colour ranges from yellow to pink to reddish brown, and is due to the inclusion of urinary pigments in the crystals. Uric acid crystals are very varied in form. Whilst rhombic prisms are the fundamental type, the crystals may occur in the form of rosettes, as plates, and be barrel shaped. Examples are shown in Fig. 23. The deposit of reddish particles has often a very characteristic appearance. Uric acid crystals

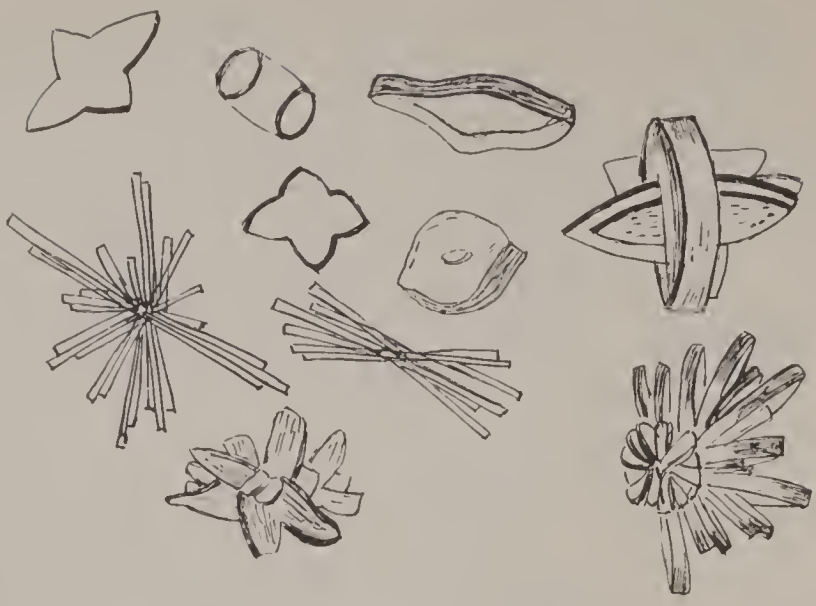

Fig. 23. Uric Acid Crystals.

dissolve in sodium hydroxide but not in acetic acid or hydrochloric acid. They are also often characterized by their property of dissolving when the urine is warmed to about 60° C. Found most frequently in the deposit of concentrated acid urines which have cooled to room temperature, uric acid crystals are not characteristic of any pathological condition. They may occur in normal people after sweating, and are found in fevers.

## Urates

Ammonium, sodium, potassium, calcium and magnesium urates are found in urine deposits. All except ammonium urates occur, like uric acid, in acid urines. Ammonium urate and sodium urate

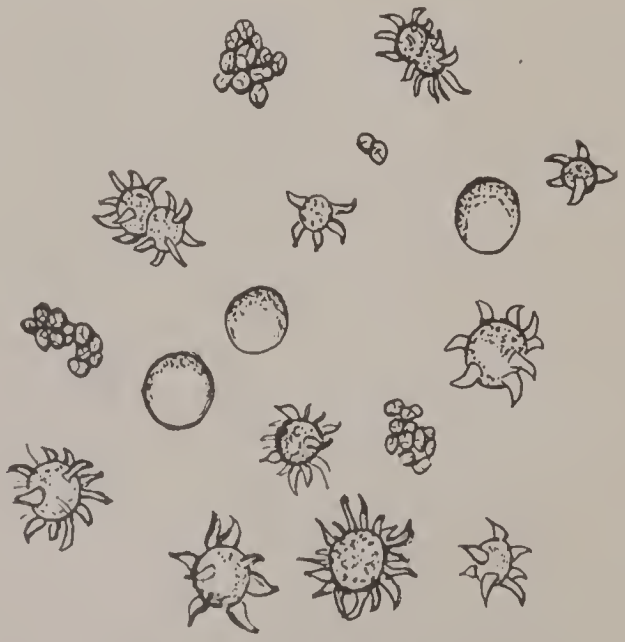

Fig. 24. Ammonium Urate Crystals.

are found as crystals, but urates often occur in an amorphous form. Urates, like the acid, are often coloured and dissolve on warming. Sodium urate may occur either as the mono or disodium salt. The acid sodium urate may form clusters of needles or stellar-shaped clusters rather similar to those of calcium hydrogen phosphate, from which they differ in solubility, and usually in being pigmented. Ammonium urate occurs either amorphous, or as crystals which are often spherical and which are sometimes covered with spikes, when they are referred to as " thorn apple " crystals (Fig. 24). These are usually pigmented. In addition they may show many bizarre shapes.

It should not be overlooked that although uric acid and urate deposits are often pigmented, this is not always the case. They may give a white deposit, which like the coloured ones, dissolves on warming.

### Calcium Oxalate

Calcium oxalate may be found in the deposit from both acid and alkaline urines. The commonest form is the octahedral, or envelope, but crystals may be ellipsoidal and then, in side-view, appear as dumb-bell shapes (see Fig. 25). While the size varies considerably, they are usually small compared with other crystals. They are not soluble in acetic acid, but dissolve in mineral acids. Calcium oxalate

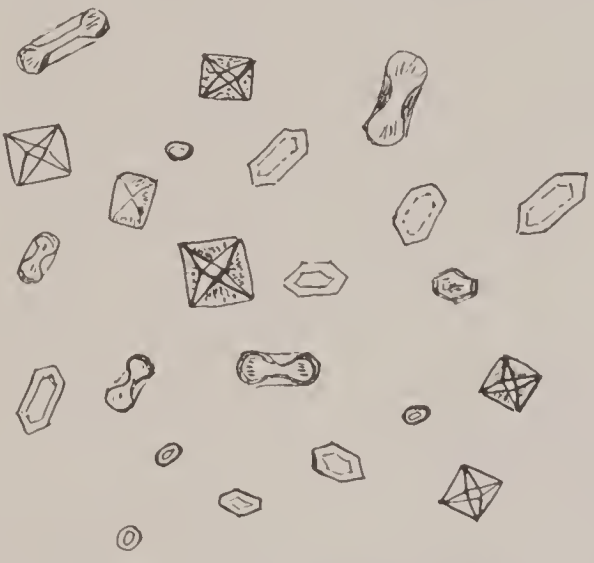

Fig. 25. Calcium Oxalate Crystals.

may be regarded as a normal constituent of urine. Whilst it is usually deposited on standing, crystals may form in the body before the urine is passed. They are also deposited on other objects present in the urine such as shreds of mucus and casts. A number of foods, particularly some fruits and vegetables, contain oxalates, notable among these being rhubarb, spinach, and strawberries. Some oxalic acid may be formed in the course of metabolism.

### Calcium Carbonate

Calcium carbonate crystals occur less commonly than the above, in human urine, and then almost always in deposits from alkaline

urines. The crystals are spherical shaped or dumb-bell in form, and are soluble in acetic acid with evolution of carbon dioxide. Calcium carbonate may also be found in an amorphous form.

### Calcium Sulphate

Crystals of calcium sulphate have been reported in acid urines as long needles, but they are very rarely seen.

### Cystine

Cystine crystals, which occur only rarely, are seen as colourless hexagonal plates (Fig. 26), which dissolve in alkalies, including ammonia, and in mineral acids, but not in water, acetic acid, alcohol, and ether. The urine, if not acid, should be made slightly so and then allowed to stand for a few hours before examining the deposit. In normal urines there is only a small amount of cystine ranging from 30 to 100 mg. daily (Dent and Rose, 1951). In cystinuria as much as $\frac{1}{2}$ to 1 gram may be excreted. Cystinuria was long regarded as being due to an inborn metabolic defect which prevents its metabolism (see Garrod, 1923). Recently Dent and Rose have shown that the increased excretion of cystine is due to a renal tubular defect so that the capacity to reabsorb cystine is impaired. The plasma level for cystine was found to be normal using a microbiological assay method, and paper chromatograms also indicated that the various amino-acids were present in normal concentrations. They showed that there is also an impaired ability to reabsorb lysine and arginine. Chromatograms were used to study these in the urine.

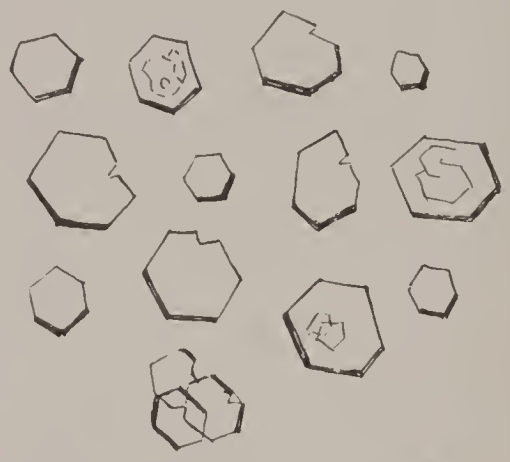

Fig. 26. Cystine Crystals.

When cystine crystals are found, confirmatory tests for solubility can be carried out on the slide. Hydrochloric acid (30 per cent. by volume) dissolves cystine crystals, but does not affect uric acid crystals, which they occasionally resemble. Tests for the presence of excess of cystine in urines in which crystals have not been seen, are as follows.

**Lead Acetate Test.** Boil about 10 to 15 ml. of urine with 2 to 3 ml. of 40 per cent. sodium hydroxide solution, and add 3 to 4 drops of saturated lead acetate solution. If cystine is present a black precipitate of lead sulphide is obtained. Urines containing much protein may give a false positive.

**Cyanide Nitroprusside Test.** Add 5 per cent. sodium cyanide to the urine to convert cystine to cysteine. Stand for a few minutes and add a few drops of sodium nitroprusside solution. Urines containing an increased amount of cystine give a magenta colour, normal urines only a faint brown.

### Leucine and Tyrosine

Crystals of these substances, only seen very rarely, may be present in cases of severe liver disease, for example, in acute yellow atrophy and sometimes in cirrhosis. Tyrosine crystallizes in sheaves or tufts of fine needles, leucine in spherical shaped crystals yellowish in colour

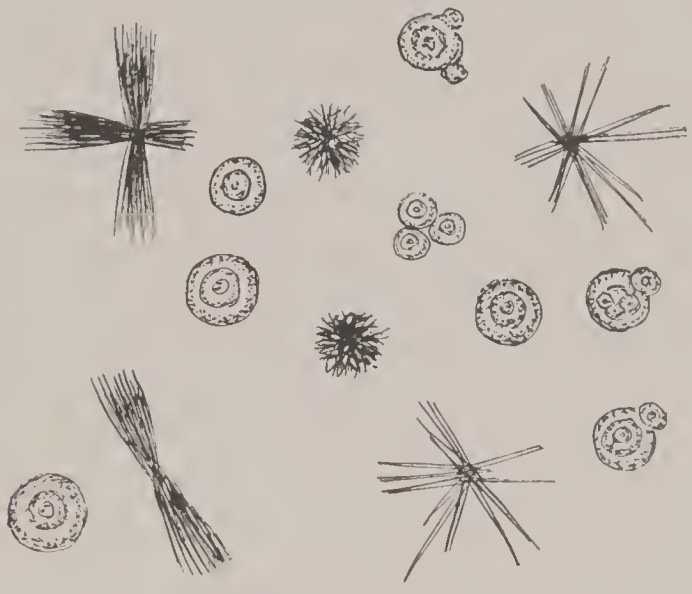

FIG. 27. Leucine and Tyrosine Crystals.

and with radial and circular striations (Fig. 27). Both are insoluble in acetone and ether but soluble in acids and alkalies. Tyrosine is only slightly soluble in acetic acid and insoluble in alcohol, whereas leucine is soluble in the former and slightly soluble in the latter.

**Cholesterol** crystals also occur extremely rarely, being found in

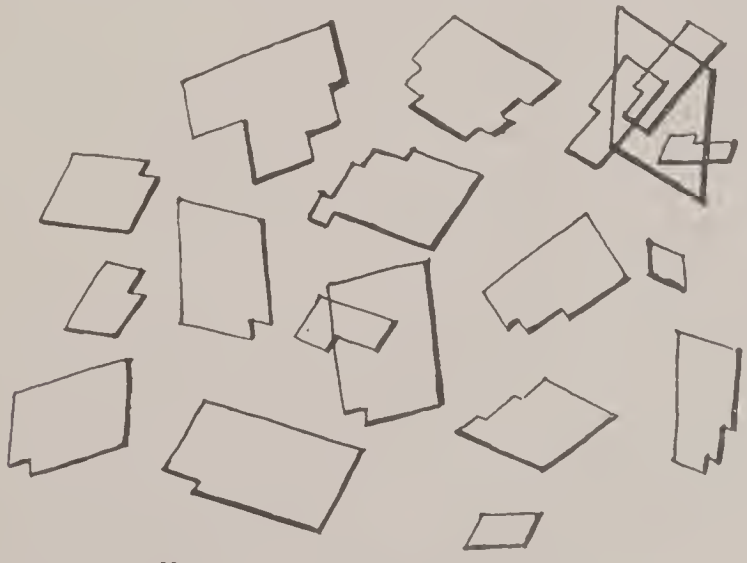

FIG. 28. Cholesterol Crystals.

some diseases of the kidneys and renal tract. The crystals are typical as rectangular or rhomboid plates with notched corners

(Fig. 28). They are soluble in ether, alcohol, and chloroform, but not in water, acids and alkalies.

**Hippuric acid** crystals are of very rare occurrence indeed. They form either colourless needles, or rhombic plates or prisms very slightly soluble in cold water and alcohol, but readily soluble in them when hot. They are insoluble in acetic acid and have no pathological significance.

**Bilirubin** crystals have been found in the deposit from acid urines containing bile pigments. They occur in tufts of fine needles or as rhombic plates, brown in colour. They are soluble in acetone.

**Indigo**, as crystals or in an amorphous form, may be found particularly in urines which have become alkaline due to bacterial contamination. It is formed by oxidation of indican. The crystals occur as small star-shaped groups of blue needles or as blue prisms. Indigo is soluble in chloroform which can be used to dissolve it out of the deposit since it may stain blue other substances present.

**Xanthine** crystals have been reported as small colourless rhombic plates, but are extremely rare. They are soluble in warm water and dilute ammonia. Unlike uric acid they do not give the murexide test (see p. 482). They have been found when xanthine calculi were present.

## Sulphonamides

When sulphonamides are being taken, crystals, usually of the acetyl derivative, may be found in the urine deposit. These may form at some point in the urinary tract, and may then cause a hæmaturia, or they may be deposited in the urine when it cools on standing. Deposition of crystals in the tubules may produce an anuria.

Such crystal formation is influenced by the solubility of the particular sulphonamide compound, by its concentration in the urine, and by the *p*H of the urine. In addition there is an appreciable personal factor. Sulphanilamide is by far the most soluble of the commonly used sulphonamides (see Gilligan *et al.*, 1943, Allott, 1947) and is very infrequently seen. This and sulphapyridine show little change in solubility with *p*H whereas sulphathiazole, sulphadiazine, sulphamerazine, and sulphamethazine (for composition see p. 502) all show a marked increase in solubility with increase in *p*H over the range of urine *p*H from 5 to 8.

The crystals are very varied in form but occur most commonly as clusters of needles. It has been suggested (see for example, Della Vida, 1947) that it is possible to recognize the sulphonamide present, from the type of crystals seen, but in many cases this is not so and as a rule is not required. The crystals often resemble uric acid crystals in shape. They may be distinguished by their solubility in acetone in which uric acid does not dissolve, by not giving a murexide test (see p. 482), and further by the colour which is obtained when both the deposit containing sulphonamides and the urine from which it was obtained are hydrolysed with acid, diazotized, and linked with a suitable coupling agent. Such a test can be carried out on

the urine by the method described for determining total sulphonamides in blood (see p. 503). In the case of the deposit, centrifuge, wash the crystals with a few ml. of cold distilled water, centrifuge again, remove the supernatant fluid, add 3 to 4 ml. of dilute hydrochloric acid and heat in a boiling water bath for half to one hour. Cool, make up to 3 to 4 ml. with water, add 0·5 ml. 0·5 per cent. sodium nitrite, stand for three minutes, add 0·5 ml. of 0·5 per cent. ammonium sulphamate solution, mix and stand for two minutes, then add 2 ml. of the coupling agent used for blood sulphonamide determination. Sulphonamides give a reddish purple colour.

A useful quick test which can be carried out on the urine from which a deposit thought to contain sulphonamide crystals is obtained is as follows (La Rosa, 1945). Prepare a solution containing 1 gram of *p*-dimethyl aminobenzaldehyde, 2 ml. of concentrated hydrochloric acid and 98 ml. of 2·23 per cent. oxalic acid. Dip strips of filter paper in this and allow to dry. One of these placed in a urine containing sulphonamides is coloured yellow.

## CELLS

Only brief reference will be made here to the different types of cells which may be found in urine deposits. For fuller accounts consult the works of Dukes (1939) and Rieder and Delepine (1899).

**Red Blood Cells.** In large numbers these give a characteristic appearance to the urine, but smaller numbers may not be recognizable naked eye or spectroscopically or even by chemical tests. According to Dukes, when red blood cells are present in numbers less than 1,000 per c.mm., only microscopical examination will detect them, which it will do down to 10 per c.mm. or less. They appear round or biconcave in shape, and yellow coloured, and have no nucleus. Their shape, however, may be very much altered by the urine in which they occur. As a result of osmosis they shrink or swell according as the urine is concentrated or dilute. Hæmolysis may also occur. Thus they may be rather difficult to recognize.

**Pus.** A few leucocytes may occur in normal urine but only in sufficient numbers to show one or two in the deposit microscopically. Any appreciable number—pyuria—indicates the presence of some inflammatory disease of the urinary tract. This is sometimes found in the absence of any demonstrable causative organism. The cells are mainly polymorphonuclears. Microscopical examination is the only satisfactory one, chemical tests being insensitive. Collection of urine from each kidney by ureteric catheterization, and from the bladder, may be used to locate the site of the pus formation.

**Epithelial Cells.** A small number of epithelial cells may be found in the deposit from normal urines, there being more in the urine of women than of men. Only large numbers are of pathological significance. Dukes has divided these into three groups, squamous epithelium, transitional epithelium, and small round or polygonal cells. The first group from the surface of the bladder, urethra or vagina, are large cells, flattish in appearance, with small oval shaped

nuclei ; the second from bladder, prostate, ureters and pelvis of the kidneys are from two to three times the size of a leucocyte. They often have a tail-like portion and are frequently seen after prostatic massage and ureteric catheterization. Finally the small round or polygonal cells, only slightly larger than a leucocyte, are rarely seen in normal urine but occur in some renal diseases.

It should be noted that abnormal cells such as tumour cells may be present. These are most commonly derived from tumours of the bladder.

**Spermatozoa.** These may be found in the urine of males for several hours after an emission of semen and may also be found after prostatic massage. They are easily recognized by their characteristic shape.

## CASTS

These are formed in the renal tubules whose shape they take. Subsequently they are pushed by the fluid along the tubules and appear in the urine. They can then be seen microscopically on examining the deposit. Classification is based on their microscopic appearance.

**Hyaline casts** are the simplest. They have a pale, transparent, homogeneous structure, are cylindrical in form, and do not contain any cells. They vary considerably in length and in breadth, the average width being that of three to four red blood cells. A few hyaline casts may be seen in the deposit from the urine of normal people even though no albumin is present. They are found in increased numbers in the majority of cases of albuminuria, and also after exercise. Hyaline casts may not be noticed if too bright an illumination is used. To avoid this, rack down the condenser and partially close the diaphragm.

**Epithelial Casts.** In cases where there is tubular damage, cells from the tubular epithelium can be taken up in the casts and so give rise to epithelial casts. In such cases the epithelial cells may be clearly recognized with the nuclei well preserved. However, degeneration may occur, giving rise to further types.

**Granular Casts.** These contain closely packed granules of varying degrees of coarseness. It has been suggested that the granules are the product of degeneration of tubular epithelial cells. It is possible to observe casts which are transitional between epithelial and granular casts, with still recognizable cells present and the remainder granular in structure. Such casts may contain a certain amount of fat. Casts which are part granular and part hyaline may also occur. True granular casts should be distinguished from hyaline casts to which a few granules from the sediment have become attached. Whilst isolated granular casts may be found in the urine of persons in whom there is no reason to suspect any renal injury, they are far less commonly found in the urine of normal persons than are hyaline casts, and in appreciable numbers their presence must be held to show the existence of a degeneration of the tubular epithelium.

**Fatty casts** are probably also derived from epithelial casts being so-called when fat globules are present, usually along with a certain amount of granular material. These are not found in deposits from normal urine and are not so common as granular casts, being only found when there is a severe degeneration of tubular epithelium.

**Waxy (colloid) casts** are composed of highly refractile substance which often has a yellowish tinge and a dullish opaque lustre. They have a tendency to split and crack. As a rule they are shorter and wider than other casts. Nothing is known of their chemical composition. Never found in normal urines they only occur when there

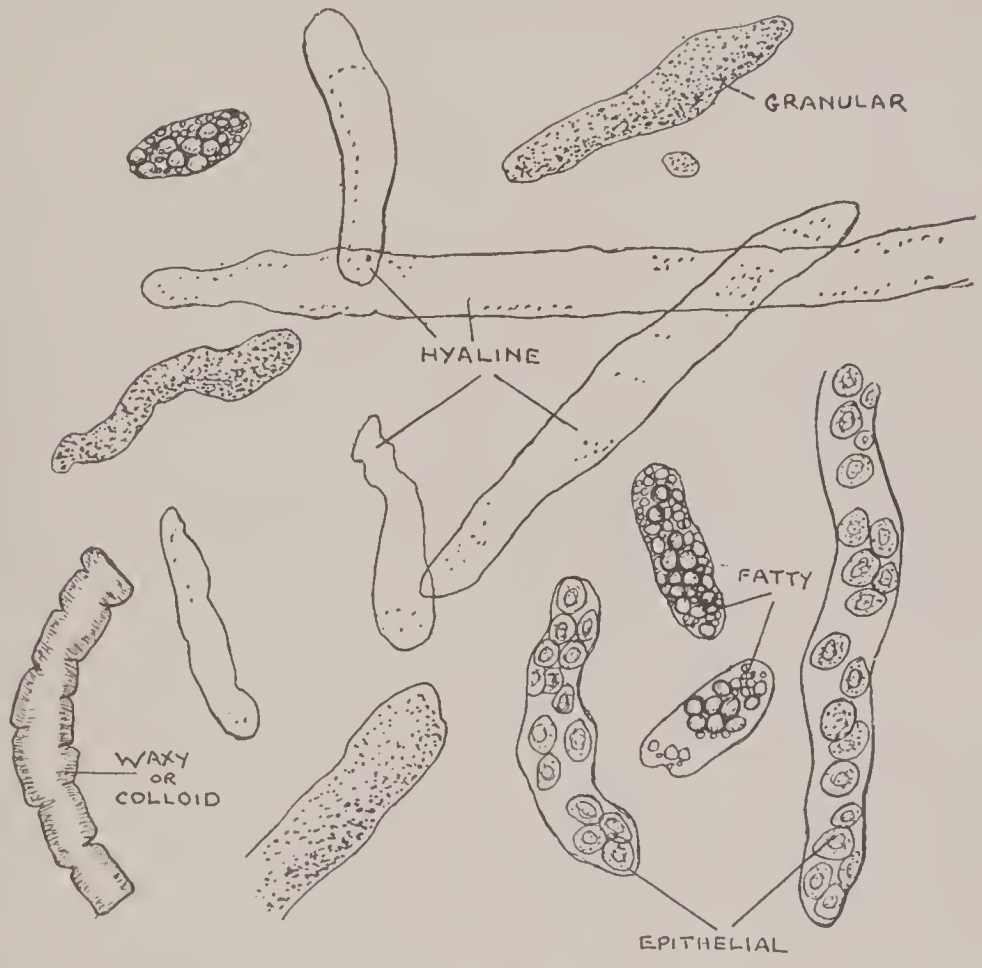


FIG. 29. Casts.

are severe long-standing changes in the tubules, and they are plentiful in amyloid disease of the kidney. Whilst acetic acid will dissolve hyaline casts, waxy casts are insoluble. These stain with methyl violet, as is characteristic of colloid material.

**Blood and Leucocyte casts** are also seen, containing, as their names imply, red blood cells and leucocytes respectively. Sometimes there is a solid mass of such cells, but more frequently a small number of cells are attached to the cast. These casts are found as a rule in the more acute phases of renal disease, for example, in acute glomerulonephritis in the early stages.

Masses of small crystals or amorphous deposits sometimes take a

shape similar to that of some casts. If doubt is felt, tests with suitable solvents can be carried out on the slide.

Drawings of the different types of cast are given in Fig. 29.

### Cylindroids

Cylindroids are pale transparent structures usually much longer and thinner than true casts. They often vary a good deal more in thickness than casts do. Casts have a fairly uniform thickness throughout their length, the sides of the cast being reasonably parallel. Further, again unlike casts, cylindroids often bend and twist. Composed of mucus, they occur occasionally in normal urine, and are increased in number in inflammatory conditions.

### Summary

The presence of some hyaline casts is devoid of diagnostic significance, and they are to be found in healthy people, particularly after exercise. Considerable numbers may be found in all diseases of the kidneys. Granular, epithelial and fatty casts all point to the presence of degenerative changes of the tubular epithelium. The largest numbers accordingly are found in subacute and chronic glomerulonephritis, that is, in the later stages of Type I nephritis, and in Type II nephritis, in Ellis's classification. They are rare in the initial stages of acute glomerulonephritis when glomerular changes only have occurred. In benign hypertension, casts are usually small in numbers or are absent, but in malignant hypertension casts of all varieties are common.

### Technique of Microscopic Examination of Urine Deposits.

Centrifuge at least 5 to 10 ml. of fresh urine. Crystals, blood, pus and casts require only a few minutes, but if bacteria are being looked for, longer periods at high speeds are necessary. After centrifuging, pour off the supernatant fluid leaving the deposit undisturbed at the bottom of the tube. Mix the deposit with the remaining few drops of urine and transfer a small amount to a microscope slide, cover with a cover glass and examine under the microscope with reduced illumination, which can be obtained by closing down the diaphragm and lowering the condenser. It is recommended that the deposit be first inspected by using the 2/3 inch objective, then more closely with the 1/6th.

**Note on Artefacts.** Quite a variety of foreign substances can be introduced into urine after it has been passed. Some of these may mistakenly be assumed to be significant. Among these substances the following are particularly important : hairs, both human and from domestic animals, hair or fibres from blankets, bits of feathers, cotton fibres, starch granules of various sorts, tea leaves, fatty or oily globules, and air bubbles. Technicians should become familiar with the appearance of these artefacts.

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*CHAPTER VIII*

# BLOOD AND URINE UREA

Urea is the main end product of protein metabolism in the body. Removal of amino groups from amino-acids, from which urea is formed, takes place in the liver.

## BLOOD UREA

Determination of blood urea is important not only in many diseases of the kidneys, but in a wide range of conditions which are not primarily renal. Most methods have used the conversion of urea into ammonium carbonate by the action of the enzyme urease found in soya beans. The ammonia formed is estimated either by nesslerization or by aeration into standard acid. Colorimetric methods have also been devised, but have not found as wide acceptance.

### Determination of Blood Urea

#### I. Urease Nesslerization Method

**Reagents.** 1. Powdered soya bean meal.

2. Isotonic sodium sulphate solution. Dissolve 3 grams of $Na_2SO_4$, $10H_2O$ in distilled water and make up to 100 ml.

3. Sodium tungstate, 10 per cent. solution of $Na_2WO_4$, $2H_2O$.

4. Sulphuric acid, 2/3 N solution.

5. Solution of Gum Ghatti. Suspend 1 gram of gum ghatti, loosely tied in muslin, for twenty-four hours in a litre of nitrogen-free distilled water. Preserve the solution so obtained with a few drops of chloroform. Discard the residue in the muslin.

6. Nessler's Reagent. Make the following two solutions.

A. Dissolve 22·5 grams of iodine in a solution of 30 grams of potassium iodide in 20 ml. of water. When solution is complete, add 30 grams of mercury. Shake well, keeping cool by immersing in cold water from time to time, until the supernatant fluid has lost all yellow colour due to iodine. Decant the supernatant fluid and test a few drops with 1 per cent. starch for the presence of iodine. If starch gives no colour add an iodine solution similar to the above, in small quantities at a time, until a slight excess of iodine can be detected on addition of a few drops to the starch solution. Dilute to 200 ml. and mix.

B. Sodium hydroxide, 10 per cent. solution accurately prepared.

To prepare the reagent mix 200 ml. of A with 975 ml. of B. Allow any precipitate to settle and use the clear supernatant fluid.

7. Stock ammonium sulphate solution. Dissolve 2·2 grams of the pure dry salt in distilled water and make up to 1 litre.

8. Standard ammonium sulphate solutions for use.

(*a*) Dilute 2 ml. of stock standard to 100 ml. with water. One ml. of this is equivalent to 0·02 mg. urea.

(*b*) Dilute 5 ml. of stock standard to 100 ml. One ml. is equivalent to 0·05 mg. urea.

**Technique.** Add 0·2 ml. of blood to 3·2 ml. of water or isotonic sodium sulphate solution. Then add about 20 mg. of powdered soya bean meal, stopper the tube, and incubate for at least fifteen minutes at 40° to 50° C. Add 0·3 ml. of 10 per cent. sodium tungstate and 0·3 ml. 2/3 N sulphuric acid. Mix well, stand for a few minutes and centrifuge.

To 2 ml. of the clear supernatant fluid add 5 ml. of ammonia-free water, or of the gum ghatti solution if trouble due to turbidity is experieneed, and 1 ml. of Nessler's reagent. Prepare standards for the colorimeter by treating 2 ml. of the standard solutions (*a*) and (*b*) similarly. For the photoelectrie colorimeter standard (*b*) is sufficient. Use a blue filter or transmission at 480 millimicrons, and read against a blank prepared by mixing 7 ml. of water and 1 ml. of Nessler's reagent. Check the reagents by putting through a eomplete reagent blank, using 0·2 ml. of water instead of blood.

**Calculation.** For the photoelectric colorimeter, since 2 ml. of supernatant fluid is equivalent to 0·1 ml. blood, and 2 ml. of standard are equivalent to 0·1 mg. of urea,

$$\text{Mg. urea per 100 ml. blood} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}10 \times \frac{100}{0{\cdot}1}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 100.$$

Since the colour may not obey Beer's law, a standard curve should bc prepared and the above formula used only if a straight line is obtained. Sueh a curve ean be prepared as follows, using a standard solution obtained by diluting 10 ml. of the stoek standard to 100 ml. One ml. of this solution is equivalent to 0·10 mg. of urea.

| Mg. urea per 100 ml. blood . . | 0 | 40 | 80 | 120 | 160 | 200 |
|---|---|---|---|---|---|---|
| Ml. standard (1 ml. = 0·10 mg. urea) | 0 | 0·4 | 0·8 | 1·2 | 1·6 | 2·0 |
| Ml. water . . . . . | 2·0 | 1·6 | 1·2 | 0·8 | 0·4 | 0 |

Develop the colour as described for the test.

With the visual colorimeter the two standards (*a*) and (*b*) eorrespond to 40 and 100 mg. per 100 ml. respectively. For values abovc 200 mg. per 100 ml. use a smaller amount of supernatant fluid.

The standard curve can also be checked by putting through some urea standards. Prepare a solution containing 200 mg. urea per 100 ml., and from this by dilution, solutions eontaining 50, 100, 150 mg. per 100 ml. Carry out urea estimation on these four solutions as described for the test.

### II. Urease Aeration Method. (Van Slyke and Cullen, 1914)

In this method the urea is converted into ammonium carbonate by the action of ureasc, and potassium carbonate added to liberate

the ammonia. This is then aerated over into a measured amount of sulphurie aeid. The volume of acid neutralized by the ammonia gives a measure of the urea present in the blood taken.

**Reagents.** 1. Phosphate buffer. Dissolve 6 grams of potassium dihydrogen phosphate and 2 grams of disodium hydrogen phosphate in water and make up to a litre. Preserve with a few ml. of chloroform.

2. Secondary octyl alcohol.
3. Powdered soya bean meal.
4. Saturated potassium earbonate solution.
5. Solid potassium earbonate.
6. N/100 sulphurie aeid. Prepare frequently from stock N or N/10.
7. N/100 sodium hydroxide (carbonate-free). Prepare frequently from stoek N or N/10.
8. Methyl red indicator.

**Technique.** Into a eylinder or tube, A. of about 100 ml. eapacity place 3 ml. blood from a pipette calibrated to contain. Wash

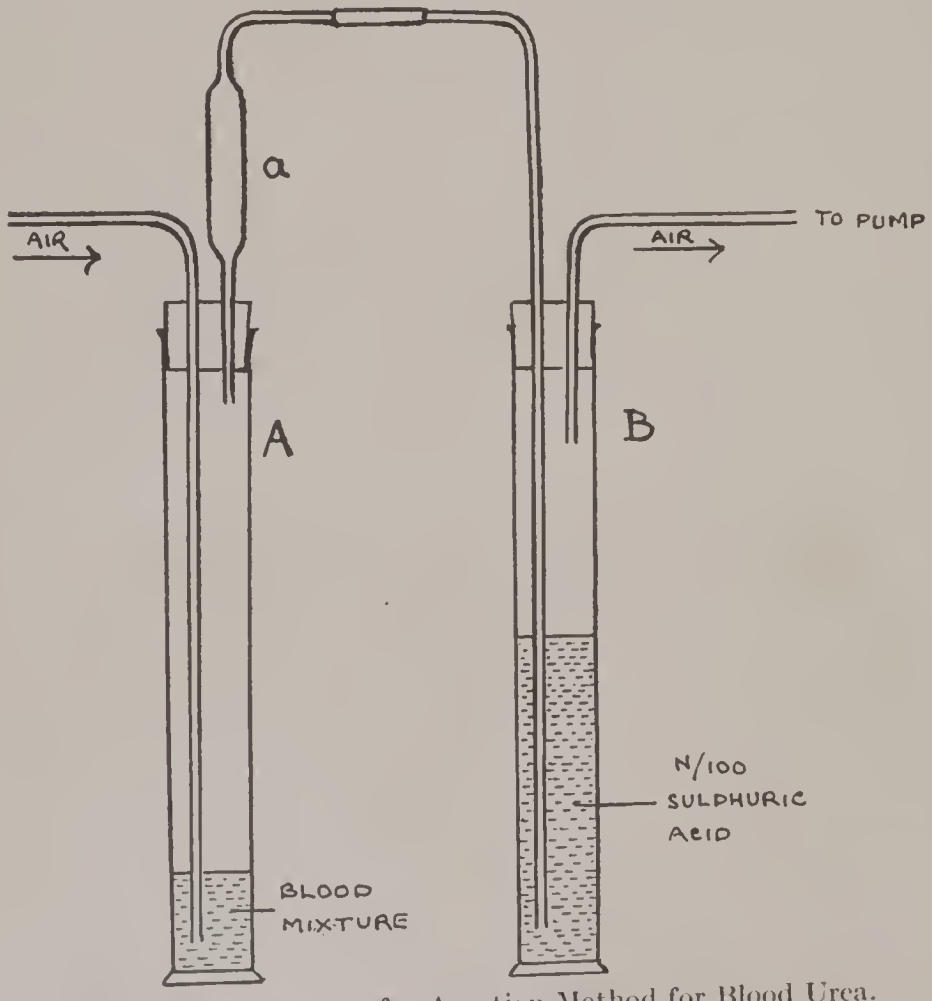


Fig. 30. Apparatus for Aeration Method for Blood Urea.

through the pipette with 5 ml. of the phosphate buffer. Add a few drops of octyl alcohol and about 0·4 gram of powdered soya bean meal. The eylinder is fitted with a rubber bung through which two glass tubes with right angle bends pass, one tube being short,

the other almost reaching to the bottom of the cylinder. Fit the tubes with short lengths of rubber tubing and plug these with short lengths of glass rod. Place the cylinder in a water bath at 45° to 50° C. for thirty minutes. During this incubation the longer tube is better raised out of the liquid in the cylinder. Into another cylinder, B, run 50 ml. of N/100 sulphuric acid. After incubating, connect the two cylinders together as shown (Fig. 30). The connection should be made by closing the rubber tube from the digestion cylinder with the fingers, removing the glass rod plug, and slipping the rubber tube on to the inlet tube of the cylinder containing the acid. This ensures that the gas in the digestion cylinder passes through the acid. Then push the longer tube down into the blood mixture. Connect the outlet tube of the acid cylinder to a pump so that air can be drawn through the apparatus in the direction shown. This air from the atmosphere should first be passed through a bottle containing 25 per cent. sulphuric acid to remove any ammonia which may be present. Insert a trap to prevent acid spray from being carried over. After connecting up, draw a little air through to clear the gas out of the digestion cylinder. Then with the pump turned off, raise the bung of the cylinder and drop in 4 grams of solid potassium carbonate, and pour in 4 ml. of saturated potassium carbonate solution. Alternatively 10 ml. of saturated solution can be used. Quickly replace the bung. Aerate, slowly at first, then more rapidly for twenty to thirty minutes if solid carbonate was used, but a little longer, forty to forty-five minutes, if only solution was added. The type of outlet tube shown, with a widened portion *a*, is used to diminish the chance of carbonate being carried over during the aeration. The author has not found this a source of error even when using straight tubing. The orifice at the end of the inlet tube in the acid-containing cylinder should be small in order to reduce the size of the bubbles. At the end of the aeration, disconnect and pipette off 25 ml. of the acid. Titrate this with N/100 sodium hydroxide using methyl red as indicator. Titrate also 25 ml. of the acid used with the N/100 hydroxide. Twenty-five ml. should be required. The two solutions should be kept equivalent to within 0·1 ml.

**Calculation.** Since a normal solution of urea contains 30 grams per litre, or 30 mg. per ml., 1 ml. of N/100 sulphuric acid is equivalent to 0·30 mg. of urea. From this it is easily seen that

Mg. urea per 100 ml. blood

$$= (\text{Titration of standard} - \text{titration of test}) \times \frac{100}{3} \times 0{\cdot}30 \times 2$$

$$= (\text{Titration of standard} - \text{titration of test}) \times 20.$$

There is a small amount of ammonia in the soya bean meal, equivalent to about 2 to 4 mg. urea per 100 ml. of blood. The method determines about 98 per cent. of the urea present. For practical purposes these two small sources of error can be allowed approximately to cancel each other out, and the calculation as given above used.

**Notes.** 1. The technique given covers blood urea values up to

500 mg. per 100 ml. For the very few higher, 75 ml. of N/100 acid can be used, multiplying by 30 instead of 20 in the calculation, or less blood used. If indicator is added to the acid before aerating, warning is given by a change in colour, that the whole of the acid has been neutralized. Further acid can then be run in, or a sufficient degree of accuracy achieved by titrating the excess of ammonia with further N/100 acid.

2. Check the apparatus by running blanks occasionally, and also put through solutions of known urea content, for example, one containing 100 mg. urea per 100 ml.

3. There is considerable scope for ingenuity in setting up a stable permanent arrangement permitting a series of determinations to be carried out simultaneously. If desired the apparatus can be arranged so as to be connected up throughout the determination, with the digestion tube immersed in water at 40° to 50° C., but on the whole this is not so convenient.

### III. Colorimetric Method using Reaction with Diacetyl (Natelson *et al.*, 1951)

**Reagents.** 1. Acid mixture. Add 75 ml. of 85 per cent. phosphoric acid to 100 ml. of water, and then to this mixture add 25 ml. of concentrated sulphuric acid.

2. Diacetyl, 5 per cent. (v/v) solution in 95 per cent. ethyl alcohol. This keeps indefinitely in the refrigerator.

3. Acid diacetyl reagent. Dilute 1 ml. of 5 per cent. diacetyl to 25 ml. with the acid mixture. Prepare fresh daily.

4. Tungstic acid reagent. Mix freshly for use, equal volumes of

(*a*) 0·15 N sulphuric acid. Add 4·15 ml. of concentrated acid to water, and make up to 1 litre.

(*b*) 2·2 per cent. sodium tungstate ($Na_2WO_4$, $2H_2O$).

5. Stock urea standard containing 100 mg. per 100 ml.

6. Standards for use. Dilute the stock standard (*a*) 1 in 100, and (*b*) 1 in 50, to obtain two standards containing 0·01 mg. per ml., and 0·02 mg. per ml. respectively.

**Technique.** Add 3·9 ml. of tungstic acid reagent to 0·1 ml. of blood. Shake well, stand for two minutes and centrifuge. To 2 ml. of supernatant fluid add 2 ml. of acid diacetyl reagent. Mix well. Treat 2 ml. of urea standard and 2 ml. of water (for the blank) in the same way. Heat the tubes in boiling water for ten minutes away from light, cool in running water and read in the photoelectric colorimeter using a blue green filter or transmission at 480 millimicrons.

**Calculation.** Since 2 ml. of filtrate are equivalent to 0·05 ml. of blood, and 2 ml. of standard (*a*) contains 0·02 mg. urea,

$$\text{Mg. urea per 100 ml. blood} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}02 \times \frac{100}{0{\cdot}05}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 40.$$

For standard (*b*) the factor is 80.

Prepare a standard curve to check the range over which Beer's law is obeyed. For this purpose a standard solution obtained by diluting the stock standard 1 in 20 can be used. Use only the straight line part of the curve.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mg. urea per 100 ml. . . . | 0 | 25 | 50 | 75 | 100 | 125 | 150 | 200 |
| Ml. standard solution (1 ml. = 0·05 mg.) . . . . . | 0 | 0·25 | 0·5 | 0·75 | 1·0 | 1·25 | 1·5 | 2·0 |
| Ml. water . . . . . | 2·0 | 1·75 | 1·5 | 1·25 | 1·0 | 0·75 | 0·5 | 0 |

Develop the colours as described for the standards above.

INTERPRETATION

The generally accepted range for the blood urea in normal persons on a full ordinary diet is from 15 to 40 mg. per 100 ml. It averages a little higher in older people in whom it may reach 50 mg. per cent. The urea content over a period is influenced by the amount of protein in the diet and tends to be lower in people on low protein diets. Thus, although the blood urea does not vary greatly during the day, it can be significantly reduced both in normal persons, and in patients with kidney disease when such diets are being taken. Hence a fall in blood urea may occur without there being any real improvement in the condition as shown by other tests such as the urea clearance test.

Urea diffuses readily into all the body fluids. Its concentration in the water of plasma and cells is the same but as the water content of the cells is a little less per unit volume than that of plasma, the ratio of urea in plasma to cells is about 5 : 4.

Blood urea is lower in pregnancy than in normal non-pregnant women. It is commonly between 15 and 20 mg. per 100 ml. and is rarely higher than 25.

*Increases* in blood urea may occur in a number of diseases in addition to those in which the kidneys are primarily involved. The same division into pre-renal, renal, and post-renal which was used in considering albuminuria is also convenient here.

1. **Pre-renal.** The most important of these are the cases in which there is dehydration with reduced volume of body fluids and so of plasma volume, usually the result of an excessive loss of chlorides. This may occur when there is severe and protracted vomiting, as in pyloric and intestinal obstruction ; in chronic intestinal obstruction without vomiting, but with accumulation of a large volume of intestinal contents ; in cases in which there is heavy loss of intestinal contents, which may occur, for example, as a result of severe and prolonged diarrhœa. Very high values for blood urea are found in these conditions if they are allowed to go untreated. Thus, in pyloric stenosis with severe vomiting the blood urea may exceed 200 mg. per cent., and even occasionally be over 300, yet can be quite rapidly brought down to normal with satisfactory treatment. The blood urea may also exceed 300 mg. per 100 ml. in ulcerative colitis with severe chloride loss. In diabetic coma, as we have seen, the blood urea may range from 50 to 150 mg. per cent., returning to

normal as soon as the coma has been treated ; in the crises of Addison's disease (hypoadrenalism) it is frequently between 50 and 100 mg. per 100 ml. and in very severe cases may be between 100 and 200.

Hæmatemesis can produce increases up to 50 to 100 mg. per 100 ml. with higher figures occasionally. Similar values may be found in shock due to severe burns, and also post-operatively. The reduced plasma volume in these conditions leads to a low blood pressure, which reduces the effective filtration rate in the glomeruli. Increased protein breakdown, which occurs in fevers and other toxic conditions, may cause moderate increases. This factor may also play a part in bringing about some of the increases referred to above. In particular, the rise in hæmatemesis and in hæmorrhage into the alimentary tract in general, may be influenced by the absorption of products from the digestion of the blood lost. Finally, in cardiac failure for example in coronary thrombosis and in circulatory collapse in any condition, there is often a moderate increase ranging from 40 to 100 mg. per 100 ml.

2. **Renal Disease.** The blood urea is naturally increased in all forms of kidney disease. In acute glomerulonephritis values from normal to over 300 mg. per cent. are seen (see p. 133). In the early stages of Ellis's Type II nephritis, corresponding to the condition known as nephrosis, blood urea is normal, but in the later stages it increases with increasing renal failure, so that in the terminal stages of this and a number of other conditions such as malignant hypertension, chronic pyelonephritis, and mercurial poisoning, it may reach 400 to 700 mg. per 100 ml.

In diseases such as hydronephrosis, congenital cystic kidneys, renal tuberculosis, conditions in which deposition of calcium is occurring as in hyperparathyroidism and hypervitaminosis D, increases are seen, the extent of which depends on the amount of kidney tissue destroyed.

3. **Post-renal diseases** which lead to increase in blood urea are those in which there is obstruction to the flow of urine. This causes retention of urine and so reduces the effective filtration pressure at the glomeruli. If prolonged, irreversible kidney damage results. Most important among these is enlargement of the prostate, in which estimation of blood urea is an essential part of the assessment of the condition. Stones in the urinary tract, stricture of the urethra, and tumours of the bladder affecting the ureters, may have a similar effect.

**Decreases in blood urea** are rare. They have been reported in some cases of severe liver disease.

### Determination of Urine Urea

It is often of value to know the urea concentration in the urine both of single specimens, and as part of tests such as the urea clearance test. The simplest method, though it is not very accurate, is the hypobromite method. The methods for estimating blood urea can be adapted for urine, but ammonia blanks have to be done and because of the very wide variations in concentration, careful dilution

may be necessary to bring the urea concentration into a range satisfactory for the test.

## I. The Hypobromite Method

The volume of nitrogen liberated by the action of hypobromite on the urea present in the urine is measured. The action is usually, though not accurately, expressed.

$$CO(NH_2)_2 + 3\ NaOBr + 2\ NaOH = N_2 + Na_2CO_3 + 3\ NaBr + 3H_2O$$

**Reagent.** Alkaline hypobromite. Slowly add 10 ml. of bromine to 100 ml. of 40 per cent. caustic soda solution, keeping cool whilst mixing. Prepare freshly for use. This gives a mixture of sodium hypobromite and sodium bromide in marked excess of caustic soda.

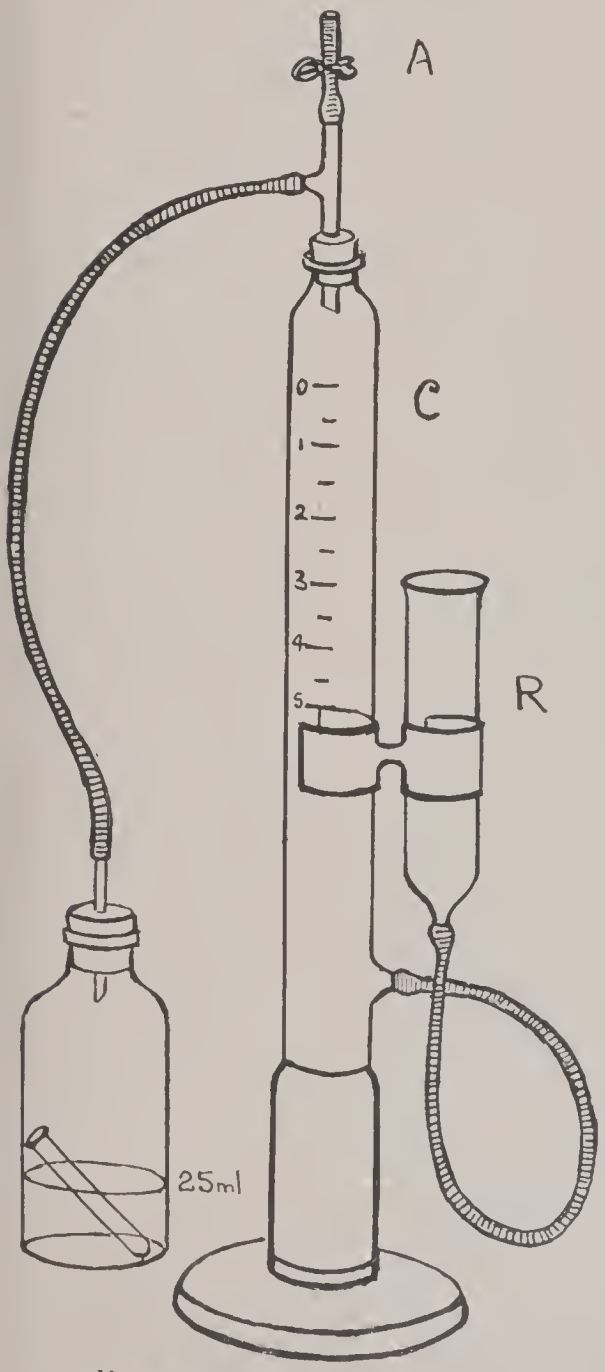


Fig. 31. Gerrard Ureometer.

**Technique.** (*a*) *Using the Gerrard Ureometer.* This is shown in Fig. 31. Place 25 ml. of the alkaline hypobromite solution in the bottle which has a calibration mark for this amount. Add a few drops of caprylic alcohol to reduce the amount of frothing. Place the small test-tube in the bottle as shown, and carefully pipette 5 ml. of the urine into it. A small piece of rubber tubing fitted on to the bottom end of this tube reduces breakages. Insert the bung into the bottle. Open clip A and bring the water in cylinder C to zero by adjusting the height of R. Close the clip. Before use test carefully for leaks by lowering the reservoir. The level of water should fall a little in C and then remain constant. Mix the urine and hypobromite carefully by gently inclining the bottle, so that frothing is moderate in amount and none of the mixture passes out of the bottle. Shake well when the reaction is nearing completion. When

evolution of nitrogen is finished, level again and read off the percentage of urea in the urine. The apparatus is calibrated so as to read directly in terms of grams urea per 100 ml. of urine.

This is not a very accurate method. Errors up to 5 per cent. above the actual urea content are common ; readings between 5 and 10 per cent. too high may be obtained occasionally. Nevertheless the method is sufficiently accurate for many clinical purposes, and because of its simplicity has been much used.

(*b*) *Maclean's Ureometer.* In this, the volume of nitrogen is

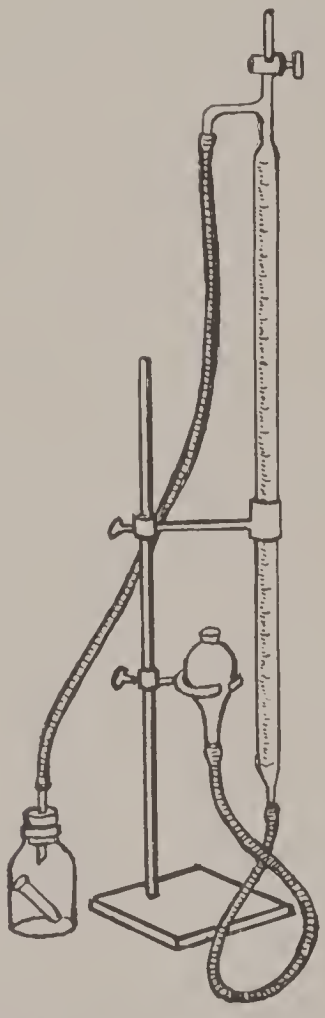

Fig. 32. Maclean Ureometer.

measured directly in ml. in a cylinder similar to a burette and calibrated in tenths of a ml. Otherwise the procedure is similar to that described above. The apparatus is shown in Fig. 32.

**Calculation.** It is found that 1 ml. of nitrogen is liberated from 2·5 mg. of urea, so if 5 ml. of urine is used,

$$\text{Grams urea per 100 ml. urine} = \frac{2{\cdot}5}{1{,}000} \times \frac{100}{5} \times \text{number of ml. nitrogen liberated.}$$

$$= 0{\cdot}05 \times \text{ml. nitrogen liberated.}$$

The effect of temperature and pressure on the volume of nitrogen can be ignored for clinical purposes.

**Notes.** 1. If no other apparatus is available an ordinary burette can be adapted for use in measuring the volume of nitrogen evolved. An extra glass tube fitted with a rubber tube and clip, for use in levelling off the level of liquid before starting the reaction, is passed through the bung of the bottle. The burette used is clamped the usual way up and fitted at the top with a cork, through which passes a glass tube connected with the bottle as in the other forms of apparatus. The lower end of the burette is then connected to the reservoir.

2. The Van Slyke gas apparatus (see p. 354) can also be used and is specially valuable if only a small amount of urine is available, as may be the case when ureteric catheterization is being done. However, in such cases it may be less trouble to use the blood urea method already in use in the laboratory, remembering that an ammonia blank has to be done with urease methods.

### II. Cole's Urease Titration Method (Modified Harwood, 1950)

The urea is converted into ammonium carbonate by the action of urease and this directly titrated with hydrochloric acid.

**Reagents.** 1. N/10 hydrochloric acid.

2. Indicator. (*a*) 0·1 per cent. bromthymol blue. (*b*) 0·1 per cent. methyl red. Mix 4 parts of (*a*) with 6 parts of (*b*). The colour changes are red at *p*H 4, orange *p*H 5, yellow *p*H 6, and green *p*H 7. Alternatively, British Drug Houses' Universal Indicator can be used.

3. Urease solution. Transfer 5 grams of soya bean meal to a 150 ml. flask and add 10 ml. of 3 per cent. potassium dihydrogen phosphate and 90 ml. of distilled water. Shake vigorously for one minute. This keeps for one month in the refrigerator.

**Technique.** Pipette 1 ml. of urine into a test tube. Add 2 drops of indicator. Adjust to *p*H 4 (red) with the hydrochloric acid. Measure 10 ml. of urease solution into another tube, add 10 drops of indicator and adjust to *p*H 4. Add 5 ml. of this to the urine tube and keep the remainder as a control. Incubate the urine tube at 40° C. When the mixture turns green add N/10 hydrochloric acid from a burette until yellow, and continue until the mixture no longer turns green. Leave at 40° C. for a further five minutes. Titrate until the mixture turns red, using the control urease solution to match.

**Calculation.** A normal solution of urea contains 30 grams per litre, so N/10 urea contains 3 grams per litre or 3 mg. per ml. Hence 1 ml. N/10 hydrochloric acid is equivalent to 3 mg. urea.

So if $n$ = number of ml. N/10 HCl required

Mg. urea per ml. urine = $n \times 3$ ; or $n \times 300$ mg. per 100 ml.

Grams urea per litre = $n \times 3$.

#### INTERPRETATION

Urine urea estimations are most commonly carried out as part of renal efficiency tests, but estimations may occasionally be made on

single specimens. A high concentration of urea shows that the kidneys possess a good concentrating power, and is not found in presence of renal failure. In cases where there is increased blood urea due to pre-renal factors, urine urea may be quite high.

The average concentration of urea over the day is about 2·0 per cent. and the total daily excretion is about 30 grams. On an ordinary diet urea nitrogen forms about 80 to 90 per cent. of the total urine nitrogen, but on a low protein diet it falls towards 60 per cent. The amount excreted may be high in conditions in which considerable breakdown of body protein is occurring.

**Note.** Fluids thought to be urine or to be contaminated with urine, can often be recognized by estimating their urea content. It may be necessary to determine the blood urea at the same time.

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*CHAPTER IX*

# CHEMICAL TESTS IN KIDNEY DISEASE

We have seen that in renal disease, failure of the kidneys to excrete urea may lead to an increase in blood urea. In the same way, other waste products accumulate, particularly nitrogenous substances, so that there is an increase also in blood uric acid and creatinine. These substances are discussed more fully later (see pp. 151, 145), but reference to their behaviour in renal disease will be made here.

**Uric Acid.** Any increase in uric acid is found to correspond roughly with the increase in urea. Although it has been stated that blood uric acid may be increased earlier than the blood urea, other factors may influence the uric acid level, so it is doubtful whether any significance can be attributed to the small increases which might be found in such cases. With increasing renal failure the blood uric acid is markedly increased from the normal of 1 to 5 mg. per 100 ml. to 10, 20, and even 30 mg. per 100 ml. Estimation of uric acid is little used in renal disease.

**Creatinine.** Whereas uric acid may possibly begin to increase before the blood urea, the reverse is true of creatinine. There is evidence that the tubules can excrete a certain amount of creatinine in man, at any rate at raised blood levels, so this may contribute to its removal from the blood. Creatinine may therefore be normal when appreciable increase in blood urea has occurred. Hence increases of creatinine are of graver prognostic significance than those of other nitrogenous substances, and the estimation has sometimes been used for this purpose. With severe renal failure, the creatinine may rise from the normal of 1·0 to 2·0 mg. per 100 ml. to over 10 mg. per 100 ml. whilst figures up to and over 30 have been found.

These substances and urea form the major part of the non-protein nitrogen of the blood, which is sometimes determined instead of the urea. There appears to be little reason for this since the urea estimation is simpler, and in almost all cases gives the same information. Non-protein nitrogen is discussed further, p. 139.

## ( RENAL FUNCTION TESTS )

Whilst the estimation of the blood urea and other nitrogenous waste products will give an index of the degree of renal impairment in the severer cases, it is possible, with lesser amounts of kidney damage, for the blood level of these substances to be kept within normal limits. In the first place the kidneys have considerable reserve capacity. In addition, in these early stages of renal impairment, a polyuria begins to develop for reasons which are not fully understood at present. As a result, the normal amount of urea and

other nitrogenous waste products can still be removed. The inability of the kidneys to excrete these in as high a concentration as in normal persons, is counterbalanced by the larger volume of urine passed. Several tests have been devised to reveal such a limitation of concentrating power and will be discussed shortly.

Furthermore, an increased proportion of the urine is passed during the night so that the ratio of day to night urine, which is normally in the region of 2 : 1 or over, falls in these early stages of renal impairment. For such a comparison, urine should be collected from 8 a.m. to 8 p.m. for the day urine, and from 8 p.m. to 8 a.m. for the night urine.

### The Specific Gravity Test (Fishberg, 1939)

This test is designed to test the concentrating power of the kidneys by producing conditions under which they should manifest as nearly as possible their maximum concentrating power. It is the simplest test to perform since it does not require any laboratory facilities. It is carried out as follows :

6 *p.m.* Give the patient a meal with a good protein content but with not more than 200 ml. of fluid to drink. *Allow no more fluid* after this meal.

Empty the bladder on retiring to rest. Discard this urine.

Discard any urine passed during the night.

On the following morning collect three specimens of urine as follows :

8 *a.m.* Empty the bladder. Specimen I.

9 *a.m.* Again empty the bladder. Specimen II. Whilst the first specimen may contain some of the night urine, this specimen should consist of urine produced by the kidneys fourteen to fifteen hours after fluid was last taken.

10 *a.m.* Collect a further specimen. Specimen III. This gives an additional check, and if the patient is getting up he may do so after passing Specimen II, so that the effect of posture on the albuminuria can be studied.

Measure the specific gravity of these three specimens. A urinometer may be used (see p. 13).

INTERPRETATION

The specific gravity of at least one specimen should exceed 1·022 and in many normal persons figures of 1·030 and over are obtained. A maximum specific gravity of less than 1·022 indicates impaired renal function and approaches nearer and nearer to 1·010 as this impairment becomes more marked.

The only possibility of false results provided the test has been properly carried out arises from evacuation of œdema fluid, particularly during recovery, when false low specific gravities may be obtained.

This simple test has proved a useful one and will show the presence of a renal defect in some cases where the blood urea is within normal limits.

**Dilution Tests.** Not only is the power of the kidneys to produce concentrated urine impaired, but also the ability to excrete dilute urines. This latter fact has been used in dilution tests. In one of these no water is taken after midnight. The bladder is emptied at 7 a.m. and 1,200 ml. of water drunk within the next half hour. The bladder is emptied hourly for the next four hours, that is, at 8, 9, 10, and 11 o'clock. The volume and specific gravity of each specimen are measured. Almost all the water drunk should be excreted within these four hours and the specific gravity of at least one should fall to 1·003 or below. With impaired renal function such a low specific gravity is not reached, and smaller volumes of urine are passed. These volumes may be less than 100 ml. with specific gravities of 1·010 in more advanced cases.

**Fixation of Specific Gravity.** It is seen that with increasing renal failure there is a contraction of the range of urine specific gravity, which in normal cases can extend from 1·040 to almost 1·000. This goes on until only urine with a specific gravity of about 1·010 can be produced. To this the term *fixation of specific gravity* is given.

It is evident that once this stage has been reached, the above tests can give no further indication as to the course of the condition. But by such times the blood urea will be increased, and the extent of this increase and the rate at which it is occurring, will provide such information. Urea clearance will also be diminished.

**Maclean's Urea Concentration Test** (McLean and de Wesselow, 1920)

This is another concentration test which has been used a good deal. Following a period of restricted fluid intake, urea is given and the urine urea determined. The ability of the kidneys to excrete a urine with a high concentration of urea is thus studied.

**Technique.** Give no food or fluid during the ten to twelve hours before the test. Early in the morning, before breakfast is allowed, say at 7 o'clock, instruct the patient to empty the bladder, and then give 15 grams of urea dissolved in 100 ml. of water. This may be flavoured with tincture of orange if desired. Then ask the patient to empty the bladder at hourly intervals for the next three hours, say at 8, 9, and 10 a.m. Measure the volume and determine the urea content of each specimen.

INTERPRETATION

A urea concentration of 2·5 per cent. is reached in most normal cases, in at least one of the specimens. If such a concentration is not reached, and particularly if the concentration fails to reach 2·0 per cent. in any specimen, some degree of renal failure is indicated. The greater the extent of this, the lower is the maximum urea concentration found. The urine urea may be less than 1 per cent. in all three specimens.

Restriction of fluid during the period before the test is essential None of the specimens should have a volume of more than 120 ml. Volumes greater than this probably indicate, either that the fluid restrictions have not been adhered to, or that œdema fluid is being

evacuated. False conclusions may then be drawn if low urea concentrations are found. The volumes of the specimens afford a good check in regard to this.

The hypobromite method for estimating the urine urea is sufficiently accurate for this test, which, because of its simplicity has been much used.

### Combined Urea Concentration and Dilution Tests

These study the kidney's ability to produce both a concentrated and a dilute urine. In the first part of the test urea is given, in the second part a considerable volume of water. One such test is described.

**Technique.** Give a light meal with not more than 200 ml. of fluid at 6 p.m. At 10 p.m. give 15 grams of urea dissolved in 100 ml. of water as in McLean's test.

11 p.m. Empty the bladder and discard the urine. The patient is encouraged to sleep throughout the night.

7 a.m. Empty the bladder and add this urine to any urine which was passed during the night. Specimen I.

7 to 7.30 a.m. Give the patient a pint and a half of fluid, which may be water, flavoured, if desired, with lemon, or weak tea.

8, 9, and 10 a.m. Empty the bladder. Specimens II, III, and IV.

INTERPRETATION

In the night urine, Specimen I, a concentration of 3 per cent. urea should be reached, whilst after drinking the fluid a urea concentration of 0·5 per cent. or less should result. When there is renal impairment the urea concentration of Specimen I is lower, that of Specimens II, III, and IV, higher than the figures given. The gap between these is progressively narrowed until it is almost non-existent. By far the greatest effect, however, is on the urea concentration of Specimen I.

### Urea Clearance Test

This is the test most widely used at the present time. It was introduced by Van Slyke and his co-workers (Möller, McIntosh and Van Slyke, 1928).

*Urea clearance* can be defined as *the number of ml. of blood which would contain the urea excreted in a minute by the kidneys.* This is given by the formula

$$\text{urea clearance} = \frac{\text{mg. urea excreted per minute}}{\text{mg. urea per ml. of blood}} = \frac{UV}{B}$$

where U = mg. urea per ml. of urine (or per 100 ml. urine).
B = mg. urea per ml. of blood (or per 100 ml. blood).
V = ml. urine excreted per minute.

It will be seen that so long as U and B are expressed in the same way it does not matter how we actually express them. It is most convenient to use mg. per 100 ml.

The normal urea clearance was determined empirically on a

number of normal people and found to average 75 ml. per minute when the rate of excretion of urine was 2 ml. or more per minute. Urea clearance was, however, found to decrease with V when V fell below 2 ml. per minute. With a urine volume of 1 ml. per minute the clearance was found to average 54 ml. per minute, and the decrease with decreasing urine volume was seen to be proportional to the square root of V. Hence for values of V from about 0·5 to 2 ml. per minute the average normal clearance is given by $54\sqrt{V}$, increasing with increase in V up to 75 when V = 2, but not increasing further. So, if we are to compare a test urea clearance with the average normal, two formulæ are required. Clearance in ml. per minute is always given by UV/B, but to compare with the normal we have to take into account this fact that the normal varies, diminishing as $\sqrt{V}$ when V is less than 2. Accordingly one formula is used for values of V under 2 and a different one when V is 2 or more.

For values of V = 2 or over, Van Slyke used the term *maximum urea clearance*. Expressing this as a percentage of the average normal we get

Maximum urea clearance (as per cent. of the average normal)

$$= \frac{\text{observed urea clearance}}{\text{average normal maximum urea clearance}} \times 100$$

$$= \frac{\frac{UV}{B}}{75} \times 100 = \frac{100}{75}\frac{UV}{B} = \frac{4}{3}\frac{UV}{B} = 1{\cdot}33\,\frac{UV}{B}.$$

For values of V less than 2, Van Slyke used the term *standard urea clearance*, since he referred these to a standard of 54 ml. per minute, the average urea clearance when V = 1. The formula for its calculation is derived as follows :

Standard urea clearance (as per cent. of the average normal)

$$= \frac{\text{observed urea clearance}}{\text{average normal standard urea clearance} \times \sqrt{V}} \times 100.$$

$$= \frac{\frac{UV}{B}}{54\sqrt{V}} \times 100 = \frac{100}{54}\frac{UV}{B\sqrt{V}} = \frac{1{\cdot}85\ U\sqrt{V}}{B}.$$

It is seen that whatever the value of V the urea clearance is finally expressed as a percentage of the average normal.

**Method of Carrying Out the Test.** The test is best carried out in the morning. Allow the patient to have breakfast as usual. Then carry out the test over a two-hour period between 9 a.m. and 12 noon. Give a drink of water, about a cupful, shortly before beginning the test. Instruct the patient to empty the bladder as completely as possible. Note the time. Discard this specimen. Get the patient again to empty the bladder as completely as possible after an interval of an hour and again an hour later. Keep these two specimens for analysis. Shortly

after collecting the first of these take a specimen of blood. Determine the urea content of the blood and urines, and measure accurately the volumes of the latter. An example of such a test as regards collection of specimens would be :

9.25 *a.m.* Cupful of water given to patient.
9.30 *a.m.* Patient empties bladder completely. Urine discarded.
10.30 *a.m.* Bladder again emptied completely. Urine Specimen I.
10.35 *a.m.* Specimen of blood taken.
11.30 *a.m.* Bladder emptied completely. Urine Specimen II.

The times need not be exactly an hour so long as they are accurately noted, though an hour is most convenient, particularly if one can arrange to begin the test exactly at the hour, which is easy with ward patients but not always so with out-patients.

Two specimens of urine are taken so as to check the reliability of their collection. It is this accurate collection of the urine specimens which is the main difficulty of the test. If sufficient water is given before the test, to promote a reasonable flow of urine, say at least 100 ml. in an hour, and if the patient is carefully instructed as to what is required, it is the author's experience with a large number of out-patients, that good agreement can be obtained between the values for the clearance from the two specimens. However, it must be admitted that without sufficient care, serious errors may result. One such error is due to the incomplete or even non-emptying of the bladder at the beginning of the test. This may lead to fantastically high figures for the first hour. In other cases the bladder may be incompletely emptied at the end of the first or second hour. If there is a serious discrepancy between the two values it is best to repeat the test. The difference between the two hourly specimens in a really satisfactory test should not exceed 10 per cent.

**Calculation.** The following will give an example of the calculation and illustrates the use of both formulæ. The test gave

Blood urea 72 mg. per 100 ml.
Urine. Specimen I (60 minutes). 1·05 per cent. urea. 96 ml.
Specimen II (60 minutes). 0·64 per cent. urea. 165 ml.

Specimen I. Applying the formula for the standard clearance

$$\text{Urea clearance} = 1{\cdot}85\,\frac{U\sqrt{V}}{B} = 1{\cdot}85 \times \frac{1050}{72} \times \sqrt{\frac{96}{60}}$$
$$= 34 \text{ per cent. of the average normal.}$$

Specimen II. Applying the formula for the maximum clearance

$$\text{Urea clearance} = \frac{4}{3}\,\frac{UV}{B} = \frac{4}{3} \times \frac{640}{72} \times \frac{165}{60}$$
$$= 33 \text{ per cent. of the average normal.}$$

Reported as urea clearance = 33 per cent. of the average normal.

**Allowance for Size. Calculation for Children.** The urea clearance naturally varies with the size of the patient. This is related to the surface area and the formulæ given can be

adapted for this by introducing instead of V, the fraction $\frac{V \times 1{\cdot}73}{A}$, where A is the surface area of the patient, 1·73 square metres being taken as the average surface area of adults so that the fraction once again becomes V for the average adult. Thus we get

$$\text{Maximum urea clearance} = \frac{4}{3}\frac{UV}{B}\frac{1{\cdot}73}{A} \text{ per cent. of the average normal.}$$

$$\text{Standard urea clearance} = 1{\cdot}85\,\frac{U}{B}\sqrt{\frac{V \times 1{\cdot}73}{A}} \text{ per cent. of the average normal.}$$

It is not usual to make this correction for ordinary adults but for very tall or small ones it may be advisable, and for children the correction should always be made.

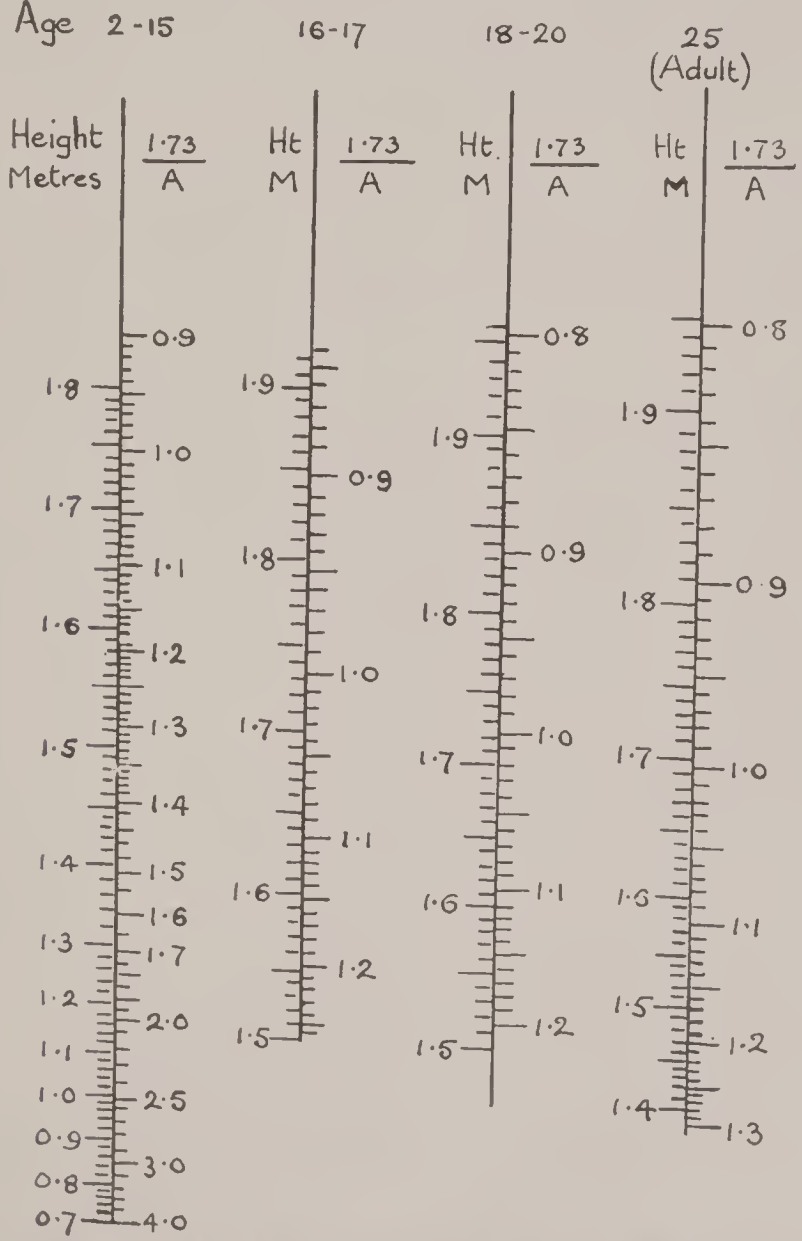


FIG. 33. Chart to correct Urea Clearances for Body Size (adapted from McIntosh, Möller and Van Slyke, *J. Clin. Invest*, 1928, 6, 467).

McIntosh, Möller and Van Slyke (1928) devised a chart from which the necessary correction could be read, if the height of the patient were known. This is given in Fig. 33.

**Urea Clearance after giving Urea.** It has been claimed that more reliable results are obtained if urea is given before the clearance test is done (see, for example, Fowweather, 1934). There is an appreciable rise in the blood urea after 15 grams of urea are taken, but it is held that the blood urea is sufficiently level during the second and third hours after giving urea for reliable results to be obtained. It is argued that more accurate urine specimens can be collected because of the diuretic effect of the urea and that more reliable results are obtained with higher blood urea levels. However, by giving water before the test a reasonable flow of urine can be produced, and it seems of doubtful value to introduce a possibly varying blood urea level. It is only when low protein diets are being taken (see below) that administration of urea appears to give appreciably higher values for the urea clearance.

INTERPRETATION

There is quite a wide range for the urea clearance in normal persons. Thus Van Slyke gave ranges of

Maximum clearance : 60 to 95 ml. per minute. Average 75.
Standard clearance (V = 1). 40 to 65 ml. per minute. Average 54.

The most commonly used dividing line between normal and abnormal is put at 70 per cent. of the average normal. One may say that the normal range extends roughly over 100 ± 30 per cent. of the average normal. The values obtained fall progressively with increasing renal failure. It is not uncommon to get values down to about 50 per cent. whilst the blood urea is within normal limits. With further fall in the clearance the blood urea is found to be raised. There is severe renal failure when the clearance falls below 20 per cent. of the average normal. Below 5 per cent. uræmic coma is present or imminent.

When the increase in blood urea is due to pre-renal causes there is a fall in urea clearance but, since the urine urea concentration is usually quite high in such conditions in contrast to the low urine urea in renal disease, the fall in urea clearance is not as a rule nearly so marked as it is in kidney disease with similar blood urea levels.

A point of some importance is that very low protein diets tend to lead to low clearance values, particularly in normal persons and in patients with milder types of renal disease (see Smith, H. F., 1949 ; Van Slyke and Dole, 1949). There are also rather wide fluctuations in urea clearance in patients with hypertension (Stanbury).

## The Phenolsulphonephthalein Test

The kidneys are able to remove from the blood a number of foreign substances when these are introduced into it. Of these the excretion of phenol red has been most frequently used as a test of renal efficiency.

**Technique of the Test.** Give the patient a cupful of water to drink

and a little later, after ten to fifteen minutes, instruct him to empty the bladder. Then inject intramuscularly 6 mg. of phenol red dissolved in 1 ml. of water. Allow ten minutes for the absorption of the dye so that the patient next empties the bladder one hour and ten minutes after the injection was given (Specimen I). Have the bladder emptied again a further hour later (Specimen II). Keep these two urine specimens separate and estimate the amount of phenol red present in each.

**Estimation of the Phenol Red.** Add 10 per cent. sodium hydroxide to the urine, gradually, until the maximum colour is developed. For the standard, measure 1 ml. of the phenol red solution used for the injection, and thus containing 6 mg. of the substance, into a litre volumetric flask, add 10 per cent. sodium hydroxide as before, to develop the colour, and make up to 1 litre with water. Depending on the amount of dye in the urine, dilute to 250 ml., 500 ml. or any appropriate volume, and compare with the standard. It is then easy to calculate how much phenol red was present in the urine specimen. Thus if the urine, when diluted to 500 ml., exactly matched the standard, the urine contained 3 mg. of phenol red and there was 50 per cent. excretion in that specimen. Rather greater accuracy is obtained if the patient's urine passed before beginning the test is used for making the standard, but the effect on the accuracy is not great compared with other factors.

INTERPRETATION

The normal excretion of dye after such an injection is

40 to 60 per cent. in the first hour,
15 to 25 per cent. in the second hour,

giving 60 to 80 per cent. excretion in the two-hour period. If intravenous injection is used, somewhat larger amounts are excreted. With increasing renal impairment progressively smaller amounts are removed from the blood. The excretion of the dye is affected by pre-renal factors, when low amounts may be passed. This test is not as reliable or sensitive an index of renal impairment as the concentration tests and is little, if any, more sensitive than estimation of the blood urea. Nevertheless it has the advantage of being a very simple test to carry out, and requires very little equipment.

## Other Blood Determinations in Renal Disease

A number of other determinations are carried out in renal disease. These are considered in detail in other chapters. Here reference will be made only to their use in renal disease.

**Serum Albumin and Globulin.** Serum *albumin* is decreased in the earlier stages of Ellis's Type II nephritis (see p. 134) when it is an important factor in causing the œdema present. In other renal conditions it is normal or but slightly reduced, except in a small proportion of cases of acute nephritis in which serum albumin is also markedly diminished. These are cases which have passed into a subacute state. Serum *globulin* is usually within the normal limits

in renal disease, but a small increase is sometimes seen when the albumin is much reduced.

**Serum Cholesterol.** This is much increased in the conditions in which serum albumin is diminished, that is, in the earlier stages of Type II nephritis (see p. 134) and in those cases of acute nephritis which pass into a subacute state. Otherwise serum cholesterol is normal, or nearly so.

**Plasma Alkali Reserve.** This may be determined when renal failure is becoming marked. It is progressively reduced as uræmic coma approaches (see pp. 135, 360).

**Serum inorganic phosphorus** is markedly increased in the later stages of renal failure (see p. 135).

**Serum calcium** is decreased in cases in which inorganic phosphorus is increased, giving rise occasionally to symptoms of tetany (see p. 297). It may also be low in those cases in which the serum albumin is much reduced.

**Plasma Chlorides.** The plasma chlorides in kidney disease are influenced by several factors. These include :

1. Impaired filtration, which tends to increase plasma chloride.
2. When tubular damage is present reabsorption of water and chloride is impaired so that a polyuria is present and is accompanied by some loss of sodium chloride which may in some cases lead to a lowered plasma chloride and a low *serum sodium*. This is particularly important in the later stages of Ellis's type II nephritis and has even occasionally been found to lead to a state of affairs similar to that found in the crises of Addison's disease (see Thorn, Kopf, and Clinton, 1944).
3. Formation or evacuation of œdema fluid. Since œdema fluid contains more chloride than plasma on account of its lower protein content, its formation tends to withdraw chloride from the plasma and vice versa.
4. Vomiting. This occurs mostly in the late stages of renal failure when uræmic coma is impending or present. It tends to reduce the plasma chloride.
5. The amount of chloride in the diet.

Accordingly the plasma chloride is rather variable in renal disease. It is sometimes increased in acute glomerulonephritis (Ellis's Type I nephritis) ; in nephrosis it is mostly normal. When renal failure is present it may be reduced and this is the commonest finding in the later stages of chronic glomerulonephritis (Ellis's Type II) particularly in uræmic coma. It should be noted, however, that plasma chloride and sodium can be maintained within normal limits for months or years, even when a considerable degree of renal failure is present (see Platt, 1950).

Plasma chloride determinations are of limited value in renal disease but may give useful information as regards the need to give or restrict salt.

### Other Clearance Tests

Recent work on the kidneys has made use of a number of clearance tests which have contributed greatly to our knowledge of the phy-

siology and pathology of the kidney. Whilst these cannot be regarded as routine tests, a fact which must be emphasized at the outset, it is felt that a description of some of these should be given.

It is now clear that the urine is formed by filtration at the glomeruli of an appreciable proportion of the plasma, the filtrate formed containing all the constituents of the plasma except the proteins and the lipids. Subsequently, during its passage down the tubules, those substances which the body needs are reabsorbed leaving mainly waste products and surplus amounts of those constituents which are of value to the body. Thus, almost all the water and glucose are reabsorbed, as are such proportions of the sodium, chloride, calcium, phosphates, etc., needed by the body to maintain the necessary blood levels, whilst the greater part of such waste products as urea escape in the urine.

It is evident that there are three possible ways in which a substance may be dealt with by the kidneys. These are :

1. Filtration at the glomeruli and reabsorption by the tubules. This is by far the commonest procedure.
2. Filtration at the glomeruli and excretion by the tubules.
3. Filtration at the glomeruli only, there being neither reabsorption nor excretion of the substance during the passage along the tubules.

Attempts were made to find a substance which is dealt with in the third way. It appears that several such substances are now available, examples being inulin, mannitol, and sodium thiosulphate. The importance of such substances lies in the fact that their clearance, defined in the same way as urea clearance (p. 118), gives a measure of the *glomerular filtration rate*, that is, of the number of ml. of plasma which are filtered at the glomeruli every minute. Thus, for example

Glomerular filtration rate (ml. per minute)

$$= \text{Inulin clearance}$$

$$= \frac{\text{mg. inulin per 100 ml. urine}}{\text{mg. inulin per 100 ml. blood}} \times \text{ml. urine passed per minute.}$$

It is found that in adults the average normal value for this is about 125 ml. per minute, which is very nearly 70 ml. for every square metre of body surface. The range has been given as 110 to 152 for men, and 102 to 132 for women (Goldring and Chasis, 1944).

When a substance is reabsorbed by the tubules its clearance is less than the inulin clearance. Thus the urea clearance averages 75 ml. when V is 2 ml. or more per minute, showing that about 40 per cent. of the urea is reabsorbed by the tubules. The relation of these two clearances, inulin and maximum urea, is in fact a fairly constant one, so that for most purposes the two can be interconverted by the formula

$$\text{Inulin clearance} \times 0{\cdot}6 = \text{Maximum urea clearance.}$$

Since glucose is almost completely reabsorbed by the tubules in normal persons, its clearance is practically nil, whilst the clearances of such valuable substances as sodium, chloride, and calcium are very low on ordinary diets.

Substances which are excreted by the tubules as well as filtered at the glomeruli have a higher clearance than inulin. These are mainly foreign substances injected into the blood, such as phenol red, diodrast, *p*-aminohippurate, the tubular excretion of the two latter being considerably greater than that of the former. Use has been made of these substances in two ways to obtain further data concerning the mode of working of the kidneys.

In the first place, at low blood levels, usually about 1 to 2 mg. per 100 ml., practically the whole of the substance may be removed in a single passage through the kidneys. The clearance of the substance is then a measure of the *effective renal plasma flow*, that is the volume of plasma passing through the kidneys per minute. This can be converted into the *effective total renal blood flow* if a hæmatocrit determination is made. Diodrast and *p*-aminohippurate clearances thus determined are found to average 600 to 700 ml. per minute (560 to 800 for men, 400 to 595 for women) so that the total renal blood flow is something like 1,100 ml. per minute.

The term *filtration fraction* is given to the ratio of the glomerular filtration rate per minute and the renal plasma flow per minute, that is, for example, to the ratio of the inulin to the diodrast or *p*-aminohippurate clearances. This gives us the fraction of the plasma passing through the kidneys which is filtered through the glomeruli. Interesting variations in this have been found, such as the observations that it is raised in hypertension and reduced in acute nephritis.

In the second place, as the plasma concentration of diodrast or *p*-aminohippurate is increased, a point is reached above which the tubules are no longer able to remove almost all the substance present as the blood passes through, so that the absolute amount excreted by the tubules then ceases to increase. At such blood levels it is therefore possible to use the excretion of these substances as a measure of the mass of functioning tubular tissue. A value for this is obtained by determining the amount of diodrast or *p*-aminohippurate excreted at such high blood levels and subtracting from this the amount filtered at the glomeruli, which can be calculated from the plasma level of the substance and the inulin clearance. This quantity has been termed the *tubular excretory mass*. The normal diodrast tubular excretory mass expressed as mg. iodine per minute averages about 53 for men (range 43 to 60·5), and 47 for women (range 33 to 52); for *p*-aminohippuric acid the average figure is 76 mg. per minute (Goldring and Chasis, 1944).

Studies of these and of the clearances of other substances such as phosphates, chloride, sodium, bicarbonate, are increasing considerably our knowledge of the behaviour of the kidneys both in health and disease.

## Technique of the Tests

Since substances such as inulin, mannitol, sodium thiosulphate, diodrast, *p*-aminohippurate, are not normally present in blood, they have to be introduced in suitable amounts if their clearances are to be determined. This has been done in two ways.

1. By continuous infusion following a priming dose. Suitable amounts of the substances used are injected intravenously to produce a satisfactory blood level, which is then maintained for the period of the test by slow continuous infusion of a further solution.

2. Alternatively, a suitable amount may be inserted into the loose tissue in the axilla from whence it is absorbed slowly, maintaining a satisfactory blood level for the period of the test.

These techniques will be briefly described together with the estimation of inulin, sodium thiosulphate and *p*-aminohippurate. The combination of inulin for glomerular filtration rate and *p*-aminohippurate for renal blood flow is the most satisfactory, the latter being preferred to diodrast on account of the greater simplicity of its estimation, which can be done by the methods used for determining sulphonamides. The combination of thiosulphate clearance and *p*-aminohippurate clearance has also been widely used.

**Method of Infusion** (Goldring and Chasis, 1944 ; Dick and Davies, 1949)

**Solutions.** 1. Inulin. A sterile 10 per cent. pyrogen-free solution is convenient. This can now be obtained from Messrs. Kerfoot, Bardsley Vale Works, Lancashire. Preparation of pyrogen-free inulin is described by Smith *et al.* (1938). The technique as there described is not easy and is best done on small amounts of inulin—about 25 to 50 grams.

2. Sodium *p*-aminohippurate solution. Twenty per cent. is used. Dissolve 20 grams of *p*-aminohippuric acid in N sodium hydroxide and make up to 100 ml. with the alkali. Adjust the *p*H to 7·2, filter through a Seitz filter and sterilize suitable small quantities (10 to 20 ml.) in the autoclave.

3. Sodium thiosulphate. Sterile 10 per cent. solution. Messrs. Boot's thiostab can be used.

4. Normal isotonic physiological saline.

From these prepare the following solutions.

5. **Priming Solution**. Thirty ml. of 10 per cent. inulin (or 18 ml. of 10 per cent. thiosulphate) + 4 ml. of 20 per cent. *p*-aminohippurate. Mix, and boil for five minutes.

6. **Maintenance Solution.** Measure 70 ml. of 10 per cent. inulin, or 54 ml. of 10 per cent. thiosulphate, and 20 ml. of 20 per cent. *p*-aminohippurate into a suitable flask, and make up to 500 ml. with normal saline and boil for five minutes.

Give smaller volumes to patients with impaired renal function, roughly in proportion to the extent of this impairment as shown by the urea clearance.

*Note.* If the substances are to be given into the axilla, add 10 ml. of 20 per cent. *p*-aminohippurate to 50 to 75 ml. of a 10 per cent. solution of inulin. Since this solution is hypertonic, it is painful. Add 1 ml. of 10 per cent. metycaine. (Procaine is not used, since it is estimated along with the *p*-aminohippurate.) Leave one hour after injecting before collecting specimens. This technique gives a

slowly falling plasma level. Collect the blood specimens at the mid-point of the periods of urine collections.

**Technique of the Test.** Give ample fluids, up to 2 pints of water, during the hour before starting the test. The patient does not need to fast. Pass a catheter, and allow the bladder to drain continuously. Insert a needle into the antecubital vein, and take a specimen of blood for use as a blank. Use some of the urine passed before starting the infusion for the same purpose. Then introduce the priming solution, taking a few minutes to do so, and follow this by the maintenance solution given at the rate of about 4 ml. per minute. After about half an hour begin collection of urine, taking three specimens over accurately timed intervals of about twenty minutes each. Withdraw blood samples at the beginning and end of each period (or alternatively halfway through). At the end of each collection period, inject 20 ml. of saline and 20 ml. of air into the bladder in order to wash out all the urine there. Include the washing in the specimen. If injection into the axilla is used, collect specimens over similar periods of time.

**Determination of Plasma and Urine Inulin** (see Bacon and Bell, 1948)

Methods have used the red colour which inulin, on hydrolysis, gives with resorcinol, or the blue colour given with diphenylamine. It is usually considered advisable to remove glucose by fermentation with yeast if the diphenylamine method is used. This is not necessary with resorcinol which is employed in the method described.

**Reagents.** 1. Acid cadmium sulphate solution. Dissolve 17·34 grams of cadmium sulphate ($CdSO_4$, $8H_2O$) in 84·55 ml. of N sulphuric acid, and make up to 500 ml.

2. Sodium hydroxide, 1·1 N.

3. Resorcinol, 0·15 per cent. in absolute alcohol. Use as pure resorcinol as possible.

4. Concentrated hydrochloric acid containing 7·5 mg. of ferric chloride per litre. The ferric chloride stabilizes the colour produced in the determination.

5. Standard solution of inulin. Prepare a stock standard containing 100 mg. inulin per 100 ml. From this prepare a standard for use by diluting 2 ml. to 100 ml. to obtain a solution containing 2 mg. per 100 ml.

**Technique.** To precipitate the proteins add 1 ml. of plasma to 5 ml. of distilled water, mix, and add 3 ml. of acid cadmium sulphate, mix, and add 1 ml. of 1·1 N sodium hydroxide. Mix well, stand for a few minutes, and centrifuge or filter. Measure 2 ml. of the filtrate into a stoppered test tube, add 3 ml. of resorcinol reagent, mix, and add 3 ml. of concentrated hydrochloric acid reagent. Mix well, and place in a water bath at 80° C. Stopper and leave for forty minutes. At the same time put up a standard, using 2 ml. of the weak standard, and a blank using 2 ml. of water. Treat these in the same way as the test. At the end of forty minutes remove from the bath, cool, and read in the colorimeter within twenty minutes after removing from

the bath. With the photoelectric colorimeter, use a blue filter, or transmission at 480 millimicrons.

**Calculation.** Since 2 ml. of standard contain 0·04 mg. of inulin, and 2 ml. of filtrate are equivalent to 0·2 ml. of plasma,

Mg. inulin per 100 ml. of plasma

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}04 \times \frac{100}{0{\cdot}2}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 20.$$

A standard curve can be prepared using a standard containing 5 mg. per 100 ml. obtained by diluting 5 ml. of the stock solution to 100 ml.

| | | | | | | |
|---|---|---|---|---|---|---|
| Mg. inulin per 100 ml. plasma . | 0 | 10 | 20 | 30 | 40 | 50 |
| Ml. standard (5 mg. per 100 ml.) . | 0 | 0·4 | 0·8 | 1·2 | 1·6 | 2·0 |
| Ml. distilled water . . . | 2·0 | 1·6 | 1·2 | 0·8 | 0·4 | 0 |

Add 3 ml. resorcinol and 3 ml. hydrochloric acid and proceed as described above.

A standard corresponding to 20 or 30 mg. inulin per 100 ml. is satisfactory for the visual colorimeter.

The usual level for plasma inulin found during the test is between 20 and 30 mg. per 100 ml.

**Urine.** It is necessary to dilute the urine. In most cases a dilution of 1 in 100 is satisfactory, but it is sometimes advisable to put up two determinations, one using 2 ml. of the diluted urine and the other 1 ml. of diluted urine and 1 ml. of water. The procedure is then exactly as described for plasma.

**Note.** The Somogyi precipitation of proteins using zinc sulphate and sodium hydroxide can be used for the plasma inulin if desired.

**Determination of Plasma and Urine Sodium Thiosulphate** (Newman *et al.*. 1946 ; Dick and Davies, 1949)

**Plasma.** Thiosulphate is oxidized by iodate to sulphate. Iodine is liberated from the remaining iodate by adding iodide, and is titrated by thiosulphate. One ml. of N/100 thiosulphate then equals 8 ml. of N/100 iodate (for full account of the reactions see Newman *et al.*).

**Reagents.** 1. Sodium tungstate, 1/3 N solution.

2. Sulphuric acid, 2/3 N.

3. N/100 potassium iodate. Dissolve 0·3567 grams of $KIO_3$ in water and make up to 1 litre.

4. Hydrochloric acid, 2 N.

5. Potassium iodide, 10 per cent. solution freshly made.

6. N/100 sodium thiosulphate. Prepare freshly from stock N/10.

7. Starch, 1 per cent. solution.

**Technique.** Add 2 ml. of plasma to 14 ml. of distilled water. Mix and add 2 ml. of 1/3 N sodium tungstate and 2 ml. of 2/3 N sulphuric acid. Stand for a few minutes and centrifuge or filter. Pipette off 10 ml. of the filtrate and add 10 ml. of N/100 potassium

iodate and 2 ml. of 2 N hydrochloric acid. Stand for at least five minutes. Add 2 ml. of 10 per cent. potassium iodide and titrate the liberated iodine immediately with N/100 thiosulphate, using a suitable microburette.

Carry out a standard titration using water instead of filtrate and put up a complete blank on blood taken before the infusion was given.

**Calculation.** Mg. thiosulphate per 100 ml. plasma
= (Titration of standard − titration of unknown)

$$\times \frac{1{\cdot}58}{8} \times \frac{10}{\text{Titration of standard}} \times \frac{100 \times 10}{\text{Ml. filtrate used}}.$$

The standard should require 10 ml. and 10 ml. filtrate are used, though larger volumes can be employed, so that

Mg. thiosulphate per 100 ml. plasma
= (Titration of standard − titration of unknown) × 19·75

Subtract the result of the blank from that of the test.

**Urine.** Iodate and iodide are added so that an excess of iodine is liberated. Some reacts with the thiosulphate and the remainder is titrated with further thiosulphate.

**Additional Reagent.** Normal sodium hydroxide.

**Technique.** Take 5 or 10 ml. of urine and make alkaline to phenolphthalein with a few drops of N sodium hydroxide. Add 25 ml. of N/100 iodate, then 2 ml. of potassium iodide and 2 ml. of hydrochloric acid. Titrate immediately with N/100 thiosulphate. Titrate 25 ml. N/100 iodate similarly as standard.

**Calculation.** Mg. thiosulphate per 100 ml. urine
= (Titration of standard − titration of unknown) × 1·58

$$\times \frac{25}{\text{Titration of standard}} \times \frac{100}{\text{Ml. urine used}}.$$

**Note.** For a method using direct titration with iodine see Brun (1950).

### Determination of *p*-aminohippuric Acid

A modification of the method for sulphonamides is used.

**Reagents.** 1. Acid cadmium sulphate. As for inulin, p. 128.

2. Sodium hydroxide, 1·1 N.

3. Hydrochloric acid, 1·2 N.

4. Sodium nitrite, 0·1 per cent. solution. Prepare freshly every few days by dissolving 100 mg. in 100 ml.

5. N-(1-naphthyl) ethylenediamine dihydrochloride, 0·1 per cent. solution. Keep in the dark.

6. Ammonium sulphamate, 0·5 per cent. solution. Prepare freshly every fortnight by dissolving 500 mg. in 100 ml. water.

7. Standard solution of *p*-aminohippuric acid containing 20 mg. per 100 ml. Dilute 1 to 100 ml. for use, to give a solution containing 0·2 mg. per 100 ml.

**Technique.** Add 1 ml. of plasma to 10 ml. distilled water, and then add 3 ml. of acid cadmium sulphate, followed by 1 ml. of 1·1 N sodium

hydroxide. Stand a few minutes, and centrifuge or filter. To 10 ml. of filtrate add 2 ml. of 1·2 N hydrochloric acid, and 1 ml. of 0·1 per cent. sodium nitrite. Shake well. Stand for five minutes and add 1 ml. of ammonium sulphamate. Stand a further three minutes, and add 1 ml. of the N-(1-naphthyl) ethylenediamine dihydrochloride. Mix well, stand for ten minutes and read in the colorimeter. As standard use 4 ml. of the weak standard solution plus 6 ml. of water and treat in the same way as the unknown. With the photoelectric colorimeter use a green filter or transmission at 520 millimicrons.

**Calculation.** Since the standard contains 0·008 mg. of *p*-aminohippuric acid, and 10 ml. filtrate is equal to 10/15 ml. of plasma.

Mg. *p*-aminohippuric acid per 100 ml.

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}008 \times \frac{15}{10} \times 100$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 1{\cdot}2.$$

A suitable standard curve can be prepared as follows :

| Mg. *p*-aminohippuric acid per 100 ml. plasma | 0 | 0·6 | 1·2 | 1·8 | 2·4 | 3·0 |
|---|---|---|---|---|---|---|
| Ml. 0·2 mg. per 100 ml. standard | 0 | 2 | 4 | 6 | 8 | 10 |
| Ml. distilled water | 10 | 8 | 6 | 4 | 2 | 0 |

Develop the colour as described for the test.

**Urine.** Dilute so as to bring the colour within the above range when 10 ml. of diluted urine is used. A dilution of 1 in 100 is usually satisfactory.

## CONGO RED TEST FOR AMYLOID DISEASE

In a number of conditions a substance known as amyloid is deposited in certain tissues. Little is known of its composition but it is held to be protein in nature. Liver, kidney, and spleen are the commonest sites for its deposition. Amyloid has a particular affinity for congo red so that if this is given intravenously in amyloid disease, it is quickly taken up. Otherwise it is slowly absorbed by the reticuloendothelial system. This property has been made the basis of a test for the presence of amyloidosis.

**Reagents.** 1. A 1 per cent. solution of congo red (Sterile).
2. Acetone.

**Technique.** Although an amount based on the body weight has been used it is satisfactory for adults to give 100 mg. as 10 ml. of a 1 per cent. solution. Inject this intravenously and four minutes later withdraw 10 ml. of blood from the other arm. After a further sixty minutes take a second specimen of blood. Allow both specimens to clot and separate the serum. Either compare the sera directly in the colorimeter or mix equal volumes of sera and acetone, centrifuge, and compare the supernatant fluids in the colorimeter. This latter procedure must be used if there is any hæmolysis. The

photoelectric colorimeter may be used, with a green filter or transmission at 520 millimicrons.

**Calculation.** The result is expressed as the percentage of the amount of dye present in the four-minute specimen still remaining in the sixty-minute one. Thus for the photoelectric colorimeter

Percentage of congo red remaining after sixty minutes

$$= \frac{\text{Reading of sixty-minute specimen}}{\text{Reading of four-minute specimen}} \times 100.$$

INTERPRETATION

In normal persons at least 60 per cent. of the dye present in the four-minute specimen should remain sixty minutes later. In amyloid disease all or almost all the dye is removed. Amyloidosis is probable if less than 40 per cent. of dye remains, almost certain if less than 10 per cent. is found. In nephrosis where there is heavy albuminuria some congo red may be lost in the urine. In such cases urine should be collected at the end of the test and examined for the amount of congo red present. In other cases little or none is lost in the urine.

Occurring mainly in chronic suppurative diseases of long duration, amyloidosis is seen mainly in chronic tuberculosis at the present time (see Lipstein, 1938). It has been found in syphilis and in Hodgkin's disease. Deposits in the kidney lead to the development of amyloid nephrosis.

## CHEMICAL FINDINGS IN RENAL DISEASE

Here we shall gather together the chemical findings so that we can see the composite chemical picture which is characteristic of the different types and phases of renal disease. Of the various classifications of nephritis, that of Ellis (Ellis, 1942 ; see also Hadfield and Garrod, 1947) is the most satisfactory at present. This distinguishes two types of nephritis, Types I and II, according to the mode of onset, which is acute in Type I and insidious in Type II. Type I corresponds to acute glomerulonephritis.

### Acute Glomerulonephritis. Type I Nephritis

Characterized by a sudden onset, this condition usually follows some streptococcal infection, and has its highest incidence in the first two decades of life.

Whilst the disease is advancing there is reduced urine volume—*oliguria*—down to about 500 ml. per twenty-four hours. Several factors may contribute to produce this. These include the œdema formation, blocking of glomeruli, vomiting, fever, cardiac insufficiency, not all of which need be present in any particular case. An increase in urine volume is generally one of the first signs of improvement.

*Hæmaturia* is present in almost every case. It may be marked for a few days or weeks. Microscopically it may be very prolonged.

*Albuminuria* is present but is not usually more than moderate in amount, at least in the acute phase.

The *specific gravity of the urine* varies widely, according to the amount of renal damage. If renal function remains normal the specific gravity may be high because of the low urine volume. In such urines the concentration of urea is also high. Both the specific gravity of the urine and the urine urea concentration fall as the concentrating power of the kidneys falls.

*Renal function* and *blood urea* may vary considerably and are not necessarily related to each other, since the blood urea is influenced by both renal and pre-renal factors. The oliguria may be sufficient, despite the high concentration of urea in the urine, to lead to appreciable nitrogen retention. Any renal failure which is present will also tend to cause a rise in blood urea. When these effects are combined, some very high values for blood urea can be found. The pre-renal is often the more important factor in causing these high blood ureas. The height of the blood urea is thus not simply an index of the renal failure. Quite high values are not incompatible with complete recovery. In a series of 160 cases Ellis found a blood urea over 50 mg. per 100 ml. in 58 (36 per cent.), and one over 100 mg. per 100 ml. in 31 (20 per cent.), with the highest value 376. Nevertheless, he only found impaired concentration tests in 15 per cent. of his cases.

In the great majority of cases the *plasma proteins* are within normal limits, the loss of albumin not being great enough to affect the plasma level appreciably. *Plasma cholesterol* is also either within normal limits or but slightly raised. The few per cent. of cases which pass into a subacute condition may, however, have quite a low serum albumin and a much increased cholesterol.

Whilst recovery is the rule (over 80 per cent.), a few die in the acute phase ; a few pass into a subacute stage in which they die, usually in a matter of months ; and the remainder make an almost complete recovery with only slight residual symptoms, which pass ultimately into a chronic glomerulonephritis, from which they may die many years later, with a chemical picture similar to that in the terminal stages of Type II. Those passing into the subacute stage show many similarities as regards the chemical picture, to patients in some stages of Type II nephritis, having considerable albumin loss in the urine, low plasma proteins, high blood cholesterol and a moderate degree of renal failure.

### Type II Nephritis

This is characterized by an insidious onset and is commoner in the middle age groups, particularly between twenty and forty. An ultimately fatal issue is the rule. The earliest abnormality appears to be a massive loss of albumin in the urine, presumably due to slight changes in glomerular permeability. The ratio of albumin to globulin in the urine is more than 10 to 1. This heavy loss of albumin reduces the plasma albumin sufficiently to produce the widespread nephrotic œdema characteristic of the first stage of this condition

clinically. This stage of Type II nephritis, which appears to be identical with the condition long known as **nephrosis,** gives a characteristic group of chemical findings. This, however, is considered by some to be a distinct disease, particularly in children.

The *urine volume* tends to be low during the development of the œdema. *Hæmaturia* is rare, but there is a large amount of *albumin* in the urine. The *urine specific gravity* is normal, or raised by the pre-renal deviation of water. At this stage *renal function* is normal, the *blood urea* is within normal limits, and concentration tests are normal. The most characteristic blood changes are the marked fall in *plasma albumin* and the large increase in *plasma cholesterol* Œdema is likely to develop with a plasma albumin below 2·5 per cent. or a total plasma protein below 5·0 per cent., but these figures are only approximate since other factors play a part. The albumin can fall well below 2·5 per cent., and the author has seen a case in which it was just under 1 per cent. The cholesterol, commonly found to be between 400 and 700 mg. per 100 ml., may even reach and exceed 1,000. The fall in protein leads to a reduced *serum calcium* but this is not significant since it does not affect the physiologically active calcium (see p. 297). Strict correlation does not occur.

Whilst the above findings are obtained in the earlier stages of the disease, a renal insufficiency gradually becomes added. With the œdema still present a moderate rise in blood urea develops, usually to between 40 and 100 mg. per 100 ml., with a moderate fall in urea clearance. Plasma albumin remains low and cholesterol high, though not on an average as high as in the earlier stages. Death from intercurrent disease, especially pneumonia, is fairly common during this stage. The condition corresponds to the chronic hydræmic nephritis of older classifications.

If the patient does not die in the above stage, the increasing renal failure with progressive atrophy of nephrons finally cuts down the loss of protein in the urine sufficiently to permit the plasma albumin level to be built up again, so that the œdema disappears. The patient passes into a state of **chronic glomerulonephritis** leading with increasing renal failure to uræmic coma. The chemical findings are similar to those obtained in the terminal stages of a number of other diseases involving the kidneys, such as **malignant hypertension, chronic pyelonephritis, mercurial poisoning, toxæmia of pregnancy.** These form a group of kidney diseases which ultimately give similar and often indistinguishable chemical and pathological pictures. In older terminology the state was known as chronic interstitial or azotæmic nephritis.

The typical features of the condition are as follows. As regards the *urine volume* there is now a *polyuria*, excretion of an increased volume of urine, with a *specific gravity* fixed in the region of 1·010. *Albumin*, though present, is usually quite small in amount, sometimes being almost barely detectable. Casts are present. The *blood urea* is increased, and whilst somewhat compensated stages seem to occur at which blood urea remains relatively stable at raised levels, often at about 100 to 120 mg. per 100 ml., ultimately there is a progressive

rise to the range 400 to 700 mg. per 100 ml., when uræmic coma is present. *Urea clearance* is much reduced and falls finally to below 5 per cent. of the average normal as uræmic coma develops.

There is an increasing tendency towards an acidosis. This is usually held to be caused by the neutralization of plasma bicarbonate by sulphuric acid and phosphoric acid resulting from protein metabolism. The failure of ammonia production by the kidneys as a means of removing acid substances is also contributory. There is accordingly a fall in plasma *alkali reserve*, so that in uræmic coma values as low as those in diabetic coma are reached, that is, down towards and under 10 volumes of carbon dioxide per 100 ml. of plasma.

Also as in diabetic coma there may be some dehydration as a result of the polyuria, and of the vomiting which frequently occurs in uræmia and which mainly removes sodium chloride, though sometimes hydrochloric acid may also be lost and thus partially counteract the tendency towards acidosis.

The increase in phosphates referred to above, is shown by the rise in the plasma *inorganic phosphorus* which commonly ranges from 5 to 15 mg. per 100 ml., occasionally reaching and exceeding 20 mg. per cent. This appears to lead to the diminished serum *calcium* also found, which in uræmic coma may be as low as 6·0 mg. per 100 ml. As a result symptoms of tetany may be observed, though the acidosis has a counteracting effect. Very occasionally the symptoms of tetany are among the most prominent clinical features of impending uræmic coma.

Plasma proteins are within normal limits or the albumin is slightly diminished, whilst the plasma cholesterol is either within normal limits or is but slightly increased. In actual uræmic coma, cholesterol may even be subnormal.

Chlorides are variable, but in the majority of cases, as uræmic coma approaches the plasma chlorides are reduced as a result of the polyuria, the vomiting, and the frequently reduced intake of salt, which more than counterbalance the effect of any retention of chloride.

**Benign Hypertension.** It may be added that in benign hypertension, in contrast to malignant hypertension, renal function is almost always normal with blood urea within normal limits or not more than slightly increased, and urea clearance also within the normal range.

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*CHAPTER X*

# NON-PROTEIN NITROGEN

The term non-protein nitrogen in blood is used to include the nitrogen from all nitrogenous substances other than protein. In urine not containing protein, it is equal to the total nitrogen. Urea is the most important of the substances which make up the non-protein nitrogen, others being uric acid, creatinine and creatine, amino acids, ammonia, and a small amount of residual or undetermined nitrogen, possibly including some peptides. Non-protein nitrogen is usually determined by a micro-Kjeldahl method after precipitating the proteins, nesslerization or aeration being used to estimate the amount of ammonia formed.

## Determination of Blood Non-protein Nitrogen

### I. Kjeldahl Nesslerization Method

**Reagents.** 1. Sodium tungstate, 10 per cent. solution.

2. Sulphuric acid, 2/3 N solution.

3. Sulphuric acid digestion mixture. Fifty per cent. (v/v), sulphuric acid in water containing 1 gram of selenium dioxide per 100 ml.

4. Nessler's reagent. Prepare as for determination of blood urea (p. 104).

5. Standard ammonium sulphate solution. Prepare a solution containing 0·1414 grams per 100 ml. Dilute 1 in 10 for use. This solution contains 0·03 mg. nitrogen per ml.

**Technique.** Prepare the Folin Wu filtrate in the usual way and pipette 1 ml. of the filtrate into a Pyrex test tube (6 × 1 inch) and add 0·2 ml. of the sulphuric acid digestion mixture. Heat very carefully over a small flame or micro-burner until the brown colour clears and the mixture becomes colourless. Continue heating carefully with a very small flame for a further five to ten minutes, cool, and transfer to a 10 ml. stoppered glass cylinder, making up to a volume of about 6 ml. Add 3 ml. of Nessler's reagent, mix, make to the mark, and again mix. Put up a standard at the same time using 1 ml. of the standard ammonium sulphate, make to about 6 ml. with water, add 3 ml. of Nessler's reagent and make to the mark as before. Compare in the colorimeter. With the photoelectric colorimeter use a blue filter or set at 480 millimicrons.

**Calculation.** For the photoelectric colorimeter, since the volume of filtrate is equivalent to 0·1 ml. blood, and the standard contains 0·03 mg. nitrogen.

$$\text{Mg. non-protein nitrogen per 100 ml. blood} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{100}{0{\cdot}1} \times 0{\cdot}03$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 30.$$

A standard curve should be prepared as follows :

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Mg. non-protein nitrogen per 100 ml. blood | 0 | 15 | 30 | 60 | 90 | 120 | 180 |
| Ml. ammonium sulphate standard (1 ml. = 0·03 mg. N) | 0 | 0·5 | 1·0 | 2·0 | 3·0 | 4·0 | 6·0 |

Treat as described for the standard above.

Put through a complete blank using water instead of blood. If this does not agree with the zero reading of the curve a correction for this should be made.

The remarks made concerning the possible non-applicability of Beer's law by this colour when discussing blood urea (p. 105) apply here so that a standard curve should always be prepared to check this point. For the visual colorimeter a standard near to the unknown should be used.

**Notes.** 1. If it is desired to use blood direct from a finger, place 0·2 ml. into 1·4 ml. water, add 0·2 ml. of 10 per cent. sodium tungstate and 0·2 ml. 2/3 N sulphuric acid. Centrifuge. Take 1 ml. of the supernatant fluid.

2. The digestion must be carried out very carefully or serious errors may arise. Overheating must be avoided. After the brown colour has cleared continue heating for about twice as long as was required for this to occur. The amount of heat used should be such that a clear colourless liquid remains at the end ; heat sufficient to produce a solid residue should not be used.

3. Trichloracetic acid can be used to precipitate the proteins. Take 1 ml. of blood, add 4 ml. of water and 5 ml. of 10 per cent. trichloracetic acid. Mix well and filter. Take 1 ml. and proceed as above.

### II. Aeration Method

The mixture after digestion can be washed into a cylinder similar to those used for the aeration method for determining blood urea. A marked excess of sodium hydroxide is added and the ammonia aerated over into N/100 acid as there described. If aeration is used more filtrate must be digested. A trichloracetic acid filtrate is convenient. Add 3 ml. of blood to 12 ml. of water and then add 15 ml. of 10 per cent. trichloracetic acid, mix well, filter, pipette off 10 ml. of filtrate (= 1 ml. blood), add 1 ml. of digestion mixture, digest as above, wash into a cylinder with three 3 to 4 ml. portions of water, and aerate into 25 ml. of N/100 sulphuric acid after adding about 4 grams of sodium hydroxide (pellets) to the digestion mixture. Titrate the whole of the acid with N/100 sodium hydroxide. Titrate also 25 ml. of the N/100 acid with the alkali. Put through a complete blank on all reagents.

**Calculation.** One ml. N/100 sulphuric acid = 0·14 mg. nitrogen.
So Mg. non-protein nitrogen per 100 ml.

= (Titration of blank − titration of unknown) × 0·14 × 100
= (Titration of blank − titration of unknown) × 14.

INTERPRETATION

The normal range for blood non-protein nitrogen is from 25 to 40 mg. per 100 ml., with very occasional values up to about 45. For plasma the range is lower, from about 20 to 30 mg. per 100 ml. Urea nitrogen forms almost half the non-protein nitrogen of whole blood. A typical division is 48 per cent. urea nitrogen, 14 per cent. amino-acid nitrogen, 4 per cent. creatine nitrogen, 1 per cent. creatinine nitrogen, 2·5 per cent. uric acid nitrogen and 30 per cent. residual nitrogen. The residual nitrogen, mainly glutathione and nucleotide nitrogen, is almost entirely in the cells. The amount of ammonia in blood is negligible.

Variations in blood non-protein nitrogen mainly reflect alterations in blood urea. Accordingly it is raised in those conditions in which blood urea is raised. As blood urea rises the urea nitrogen forms an increasing percentage of the non-protein nitrogen, so that in uræmic coma with the blood urea at 500 mg. per 100 ml., it forms about 90 per cent. In most cases determination of non-protein nitrogen gives the same information as blood urea, but is less convenient to do. Most workers prefer to use the blood urea.

In very severe liver disease, since there is a tendency for the blood urea to be low, and for the amino acid content of the blood to be increased, the urea may form a smaller proportion of the non-protein nitrogen.

## Determination of Urinary Total Nitrogen

Use of nesslerization of the Kjeldahl digest is not so satisfactory for urine, so that a Kjeldahl distillation method is most often used. The Kjeldahl aeration method has also been employed, and may be more convenient for some laboratories. This and the micro and macro Kjeldahl distillation methods will be described.

### 1. Kjeldahl Aeration Method

**Reagents.** 1. Concentrated sulphuric acid.
2. Potassium sulphate.
3. N/100 sulphuric acid.
4. N/100 sodium hydroxide.
5. Sodium hydroxide. Forty per cent. solution or solid pellets.
6. Methyl red indicator.

**Technique.** Dilute the urine 1 in 10, and pipette 2 ml. of this into a Pyrex test tube. An 8 × 1 inch tube is most suitable. Add 1 ml. concentrated sulphuric acid and 0·2 gram of potassium sulphate. Heat over a small flame so that the liquid boils gently. The mixture becomes dark brown and then clears, becoming almost colourless. Continue heating for a further ten to fifteen minutes. Allow to cool. For the aeration, the apparatus described for the aeration method (p. 106) may be used. Wash the digestion mixture into a cylinder with three portions of about 3 ml. of distilled water. Connect to a cylinder containing 50 ml. N/100 sulphuric acid. Add 8 to 10 ml. 40 per cent. sodium hydroxide or about 4 grams of the solid pellets,

to the digestion mixture. Draw a current of air through, bubbling slowly for a few minutes at first, then rapidly for about twenty minutes. Titrate 25 ml. with N/100 sodium hydroxide. Carry out a blank on all the reagents.

**Calculation.** One ml. N/100 sulphuric acid is equivalent to 0·14 mg. of nitrogen. Hence

Urine nitrogen per 100 ml. of urine = (ml. titration of blank − ml. titration of test) × 2 × 0·14 × $\frac{100}{\text{volume of urine used}}$.

So, if 2 ml. of urine diluted 1 in 10 were used

Urine N in mg. per 100 ml. = (ml. titration of blank − ml. titration of test) × 140.

Grams urine nitrogen per diem = (ml. titration of blank − ml. titration of test) × 1·4 × $\frac{\text{volume of twenty-four hour specimen}}{1{,}000}$.

### 2. **Micro-Kjeldahl Distillation** (Pregl)

After digesting as described in the above method, wash the mixture into the vessel C of the distillation apparatus (Fig. 34) with three or

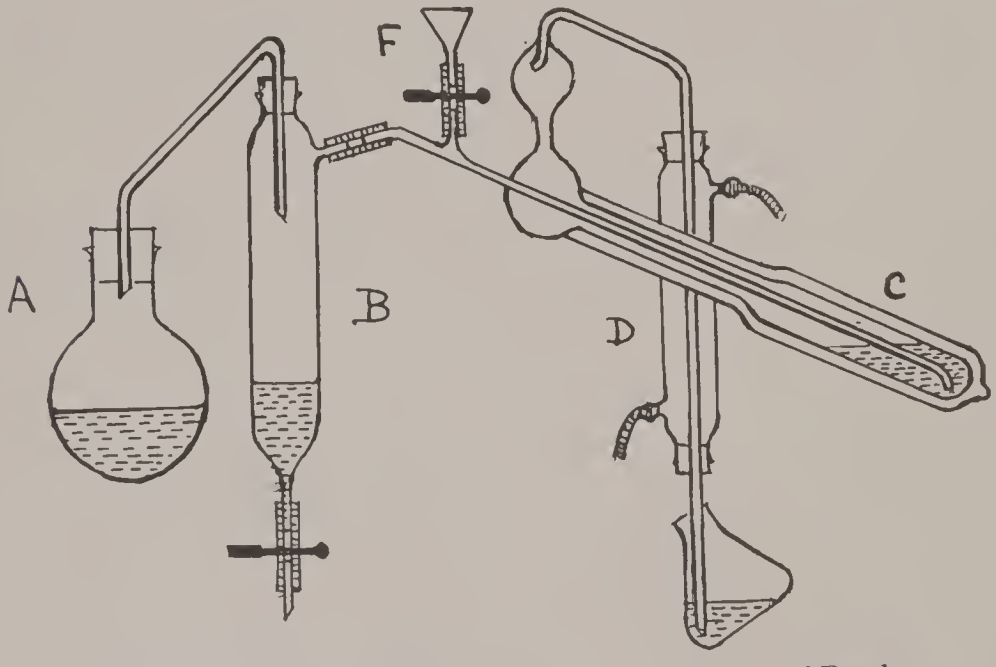



Fig. 34. Micro-Kjeldahl Distillation Apparatus of Pregl.

four washings of 3 to 4 ml. of water. Then run in 12 ml. of 40 per cent. sodium hydroxide down the funnel F. Boil water in the flask A and pass the steam through the mixture in C. Carrying the ammonia with it, the steam is condensed in D, the tube of which dips beneath the surface of 10 ml. of N/50 acid. Pass the steam for five to ten minutes. Then lower the receiver a little so that the end of the condenser tube is above the surface of the liquid in the flask and pass steam for a further two minutes. At the end of this time wash the liquid on the end of D into the receiver, and titrate the contents with N/100 sodium hydroxide. Carry through a blank.

**Calculation.** The calculation is as before except that the factor 2 is omitted since in this case the whole of the acid is titrated and not half as in the aeration. So

Grams urine nitrogen per diem = (ml. titration of blank − ml. titration of test) × 0·7 × $\frac{\text{volume of twenty-four hour specimen}}{1{,}000}$.

For very concentrated urines use 1 ml. of urine diluted 1 in 20.

**Notes on the Use of the Apparatus.** Before use the apparatus should be steamed out thoroughly. Pass steam for about ten minutes without passing water through the condenser. Empty the distillation flask C by ceasing to heat the water in A so that the contents are then sucked back into B. Before introducing the digestion mixture wash out again with distilled water. Steam out the receiving flask before use. Empty the distillation flask in the same way at the end of the test and wash out similarly.

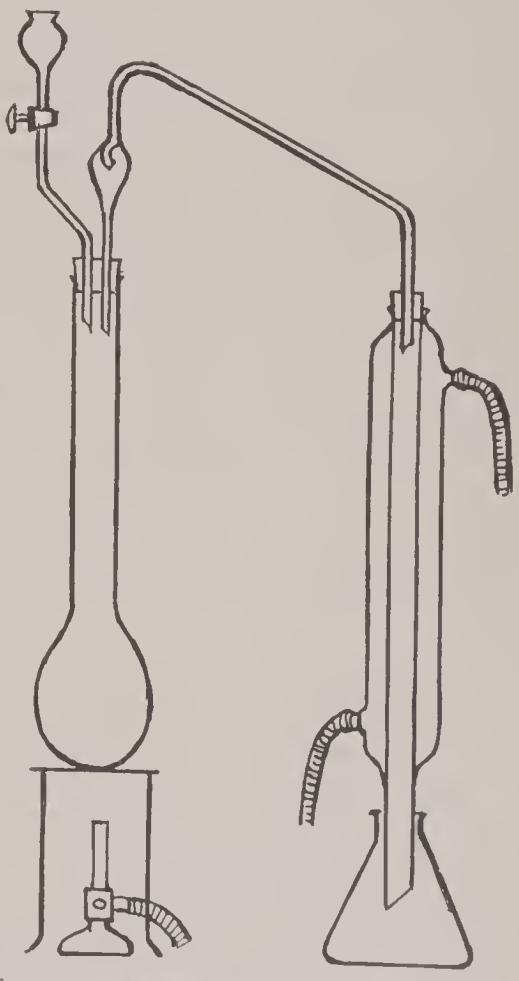


FIG. 35. Macro-Kjeldahl Distillation Apparatus.

### 3. Macro Kjeldahl Distillation

This may be employed using 5 ml. of urine. Place in a 300 ml. Kjeldahl flask and add 10 ml. of concentrated sulphuric acid and 0·2 gram of copper sulphate. Heat moderately through the usual stages and for an hour after the colour has cleared. Cool and add about 150 ml. of distilled water (nitrogen free). Set up as shown in the diagram (Fig. 35). and run in an excess of 40 per cent. sodium hydroxide from the separating funnel. Make sure an excess has been added by adding 2–3 drops of indicator (phenolphthalein) to the liquid in the flask. Distil the ammonia over into 50 ml. of N/5 sulphuric acid continuing until the volume of liquid in the Kjeldahl flask has been reduced by about a half. Titrate the remaining acid in the receiving flask with N/10 sodium hydroxide.

**Calculation.** One ml. of N/10 sodium hydroxide is equivalent to 0·0014 gram of nitrogen. If 5 ml. of urine are used,

Grams urine nitrogen per diem = (ml. titration of blank − ml. titration of unknown)

$$\times\ 0{\cdot}0014 \times \frac{\text{volume of twenty-four hour specimen}}{5}.$$

INTERPRETATION

The daily urinary nitrogen output is greatly influenced by the amount of protein in the diet. On an average diet some 14 to 15 grams are excreted, with a normal range from 11 to 18 grams. It is much lower on protein-poor diets when it may fall to below 5 grams, whereas on a high protein diet it ranges from 20 to 30 grams. In a normal adult on an adequate diet the total nitrogen excreted in the urine and fæces is almost equal to that taken in the food. The fæces contain only a small part of this nitrogen, about 90 per cent. of the nitrogen lost being found in the urine. In metabolic studies this figure may be used, fæcal nitrogen estimations dispensed with, and total nitrogen excretion calculated from urine nitrogen alone. It has been argued, however, that fæcal nitrogen is not related to food nitrogen and that it is more accurate to use a daily fæcal nitrogen of 1·3 grams. For really accurate work analysis of the fæces is necessary. For further discussion of fæcal nitrogen, see p. 282.

On ordinary diets the urea nitrogen forms 80 to 90 per cent. of the total urine nitrogen whereas on a low protein diet it may fall to about 60 per cent. The absolute amounts of ammonia and creatinine remain practically unchanged and there is only a relatively small drop in the uric acid excretion. Accordingly these form a much larger percentage of the reduced nitrogen excretion on a low protein diet. Thus Folin (1917) found

| | Total N | Urea N | Ammonia N | Uric acid N | Creatinine N | Undetermined N |
|---|---|---|---|---|---|---|
| | grams | per cent. | per cent. | per cent. | per cent. | per cent |
| Ordinary diet : 19 grams N . . | 16·8 | 87·5 | 3·0 | 1·1 | 3·6 | 4·9 |
| Low protein diet : 6 grams N . . | 3·6 | 61·7 | 11·3 | 2·5 | 17·2 | 7·3 |

It should be pointed out that whilst a person is in nitrogen equilibrium on an ordinary diet, this is not the case on low protein diets. There is then a negative nitrogen balance, more nitrogen being excreted than is being taken in the diet. The minimum amount of protein required to give a balance is probably between 0·5 and 0·7 grams per kilogram of body weight, that is for an average male adult between 35 and 40 grams daily, equivalent to about 6 grams of protein N. Above such a figure nitrogen equilibrium should be established.

Urine nitrogen estimations are only rarely done and then almost always as part of metabolism experiments.

The above discussion has assumed the urine to be free from protein. In metabolism work any such protein would naturally be included in the urine nitrogen.

## CREATINE AND CREATININE

The method commonly used for the estimation of creatinine makes use of the Jaffé reaction, the production of a red colour with an

alkaline picrate solution. This reaction is not specific for creatinine and there are other substances in blood, more especially in the red blood cells, which also give this colour. However, " chromogen " thus estimated and expressed as creatinine has clinical significance. Bonsnes and Taussky made a useful study (1945) of the best conditions for the development of this colour. Estimation of these substances is most commonly made on urine.

**Determination of Creatinine in Urine. Alkaline Picrate Method**
Bonsnes and Taussky, (1945)

**Reagents.** 1. Picric acid, 0·04 M solution (9·16 grams per litre). Picric acid as supplied contains added water. It can be dried between filter papers and the picric acid purified (Benedict, 1922, 1929) by crystallization from acetic acid if the colour developed on the addition of alkali gives a colorimeter reading more than twice that of the picric acid solution alone. For this purification dissolve about 100 grams of dried picric acid in 150 ml. of glacial acetic acid in a conical flask and heat to boiling on an electric hot plate. Filter into a dry beaker through a fluted funnel. Cover and allow to stand overnight, seeding with a few small crystals of pure picric acid if necessary. Filter, using suction, and wash with 30 to 40 ml. of cold glacial acetic acid. Suck as free from the acetic acid as possible and dry at 80° to 90° C. until there is no odour of acetic acid.

High grade analytical reagent is satisfactory without further purification.

2. Sodium hydroxide, 0·75 N solution.

3. Stock solution of creatinine containing 1 mg. of creatinine per ml. Dissolve 1 gram of pure dry creatinine in N/10 hydrochloric acid and make up to a litre with the acid. This solution keeps almost indefinitely.

4. Standard creatinine solution for use. Prepare from the above by diluting 1 ml. of stock solution to 100 ml. with water. This contains 0·01 mg. of creatinine per ml.

**Technique.** Dilute 5 ml. of urine to 500 ml. in a volumetric flask. Pipette 3 ml. of the diluted urine into a test tube and add 1 ml. of 0·04 M picric acid followed by 1 ml. of 0·75 N sodium hydroxide. Treat 3 ml. (= 0·03 mg.) of the standard solution for use in the same way and put up a blank consisting of 3 ml. water, and 1 ml. of each of the reagents. Allow to stand for fifteen minutes and then read in the colorimeter during the next half hour. If the photoelectric colorimeter is used, use a blue-green filter, or transmission at 500 millimicrons.

**Calculation.** Since the standard contains 0·03 mg. creatinine, and 3 ml. of diluted urine corresponds to 0·03 ml. of the original urine, for the photoelectric colorimeter we have

Grams creatinine per litre of urine

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}03 \times \frac{1000}{0{\cdot}03} \times \frac{1}{1000}.$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}}.$$

Since Beer's law may not be followed sufficiently closely, prepare a standard curve to check this. For this curve dilute 2 ml. of the stock standard to 100 ml. to obtain a solution which contains 0·02 mg. per ml. Set up tubes as follows :—

| | | | | | | |
|---|---|---|---|---|---|---|
| Grams per litre of urine . . . | 0 | 0·4 | 0·8 | 1·2 | 1·6 | 2·0 |
| Mg. per 100 ml. urine . . . . | 0 | 40 | 80 | 120 | 160 | 200 |
| Ml. of standard solution (0·02 mg. per ml.) | 0 | 0·6 | 1·2 | 1·8 | 2·4 | 3·0 |
| Ml. distilled water . . . . | 3·0 | 2·4 | 1·8 | 1·2 | 0·6 | 0 |

Add picric acid and sodium hydroxide as above.

For the visual colorimeter the standard given above is usually satisfactory. In all cases a standard giving a colour nearly the same as that given by the test should be used.

### Determination of Creatine in Urine

To determine creatine in urine it is necessary to estimate the creatinine present before and after heating with acid to convert the creatine into creatinine. From the difference the amount of creatine can be calculated.

**Reagents.** As for the determination of creatinine.

**Technique.** Pipette 3 ml. of the urine diluted 1 in 100 as above, into a 6 × $\frac{1}{2}$ inch test tube, and add 1 ml. of 0·04 M picric acid. Mark the test tube at the level of the liquid and place in a beaker of briskly boiling water for forty-five minutes. At the same time place a blank containing 3 ml. of water and 1 ml. of the picric acid in the water. At the end of forty-five minutes cool to room temperature, make up to the mark (4 ml.) with distilled water, so as to make good any loss due to evaporation. Then add 1 ml. of 0·75 N sodium hydroxide and complete the estimation as described for creatinine. The reading on the colorimeter now includes the creatinine originally present plus that formed from any creatine present in the urine. The difference between this and the creatinine determined as above, gives the creatinine formed from creatine.

**Calculation.** One gram of creatinine is formed from 1·16 grams of creatine. Subtract the preformed creatinine from the total creatinine after heating with acid, and multiply by 1·16.

### Determination of Creatinine and Creatine in Blood

The method of Bonsnes and Taussky has been applied to blood by Brod and Sirota (1948).

#### Determination of Creatinine

A modified Folin Wu tungstic acid method is used for precipitating the proteins. Estimation is then as above.

**Reagents.** 1. Sodium tungstate, 5 per cent. solution. Dissolve 5 grams of $Na_2WO_4 . 2H_2O$ in water and make up to 100 ml.

2. Sulphuric acid, 2/3 N solution.

Picric acid and sodium hydroxide as above.

**Technique.** Dilute 2 ml. of plasma with 2 ml. of distilled water, and precipitate the proteins by adding 2 ml. of 5 per cent. sodium tungstate and 2 ml. of 2/3 N sulphuric acid, added, drop by drop, with constant shaking. Stand for ten minutes and filter.

Pipette 3 ml. of the filtrate (= 0·75 ml. plasma) and add 1 ml. of the picric acid and 1 ml. of sodium hydroxide. Read in the colorimeter after standing for fifteen minutes. Carry out a complete reagent blank substituting water for the plasma. As standard, 1·5 ml. of the standard for use (p. 143), containing 0·015 mg. creatinine, and 1·5 ml. water, put up similarly, is satisfactory.

**Calculation.** For the photoelectric colorimeter, using the standard suggested.

$$\text{Mg. creatinine per 100 ml. blood} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}015 \times \frac{100}{0{\cdot}75}.$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 2{\cdot}0.$$

The standard curve given above for creatinine in urine can be used. The dilution of the plasma is 1 in 4 compared with 1 in 100 for the urine, so if the same standard solution is used the corresponding plasma levels are

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mg. creatinine per 100 ml. plasma. | 0 | 0·8 | 1·6 | 2·4 | 3·2 | 4·8 | 6·4 | 8·0 |
| Ml. standard solution (0·02 mg. per ml.) . . . . . | 0 | 0·3 | 0·6 | 0·9 | 1·2 | 1·8 | 2·4 | 3·0 |
| Ml. water . . . . . | 3·0 | 2·7 | 2·4 | 2·1 | 1·8 | 1·2 | 0·6 | 0 |

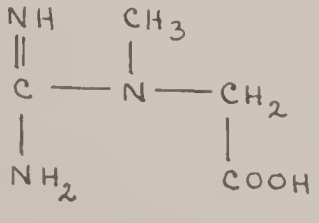
It is advisable to increase the number of points on the curve for lower creatinine concentrations. For higher values of creatinine use less filtrate. The remarks made about Beer's law under urine creatinine apply here.

### Determination of Creatine

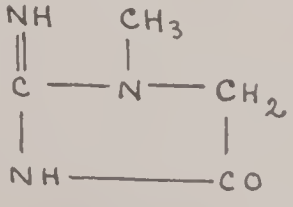
**Technique.** Take 3 ml. of the protein-free filtrate prepared as above, and add 1 ml. of the picric acid solution. Heat for forty-five minutes in boiling water as before, make up to 4 ml. afterwards, and develop the colour as previously. As with urine the creatinine present before heating is subtracted from the total after heating, and the figure obtained converted into creatine by multiplying by the factor 1·16.

INTERPRETATION

The formulæ for creatinine and creatine are as follows :




Creatine




Creatinine

Creatinine is thus the internal anhydride of creatine, being formed when water is removed. This can be effected by boiling with hydrochloric acid or picric acid.

**Urine.** Creatine is necessary in the process of muscle contraction, which is related to the amount of phosphocreatine broken down, resynthesis occurring after contraction. Creatinine is derived from creatine and is a waste product. The *normal daily excretion of creatinine* ranges from 1 to 2 grams, being as a rule nearer to the higher limit in men and to the lower in women. Creatinine is largely endogenous in origin, so that the amount excreted in the urine is little influenced by the diet, provided this is a normal one and does not contain considerable amounts of creatine or creatinine rich foods such as meat. Such endogenous creatinine is related rather to the amount of phosphocreatine in the body.

There is little if any *creatine in the urine* of adults, particularly in the case of males, but some may be found in the urine in pregnancy, and for a short time after delivery, and also in childhood. Under certain abnormal dietary conditions it may occur in the urine of adults, for example, when a low carbohydrate diet is being taken, and in fasting, when it is presumably derived from the breakdown of muscular tissue. It has been reported in increased amounts in the urine of athletes during training.

The sum of the milligrams of creatinine and creatine nitrogen excreted per kilogram of body weight has been termed the *creatinine coefficient*. It ranges from about 7 to 10 for men and from 5 to 8 in women, and may be regarded as a relative measure of the functioning muscular tissue. There are varying opinions as regards the effect of exercise on the creatinine coefficient, some workers holding it to be increased, whilst others do not. The factors for converting creatinine and creatine into their nitrogen content are 0·372 and 0·321 respectively.

Interest in pathological conditions is mainly concerned with the excretion of creatine, particularly in muscular disorders. Increases are often, though not consistently, found in such conditions as myasthenia gravis, muscular atrophies, and the different types of myositis. They also occur in starvation and in fevers where there is considerable muscular wasting, and sometimes, for the same reason, in diabetes mellitus. The increased metabolism of hyperthyroidism has a similar effect.

A *creatine tolerance test* has been used (see Milhorat and Wolff, 1936; Richardson and Shorr, 1935). When 1·32 grams of creatine hydrate are taken by a normal adult, not more than 30 per cent. of it is excreted in the urine in the following twenty-four hours. A figure of not more than 20 per cent. has been suggested for men. A greater excretion than this (diminished creatine tolerance) must be taken to indicate the presence of a pathological creatinuria. It is markedly increased in those conditions in which there is increased creatinuria. Some degree of abnormality may be found in cases not severe enough to show appreciable increase in creatine excretion. Creatine tolerance then, may be diminished in the various muscular disorders, most

markedly in the dystrophies. It is also diminished in hyperthyroidism. It may be increased in hypothyroidism.

In conditions where there is an increased excretion of creatine, so long as this is small, the creatinine plus creatine is not affected. However, as the amount of creatine lost increases, even though creatinine excretion tends to fall, the excretion of the two together rises so that the creatinine coefficient also rises. In some severe muscular dystrophies this rise may be considerable.

**Blood.** The Jaffé reaction used in the estimation of creatinine is so unspecific that there has been considerable doubt and disagreement as to how much of the colour produced in the case of blood was actually due to creatinine. However, recent work suggests that by far the greater part, if not all, of the chromogenic substances in plasma is actually creatinine, though in the cells it is rather less than half. Measured by the above method, the normal *blood creatinine* is between 1 and 2 mg. per 100 ml., though in whole blood the concentration of true creatinine may be little more than half that amount. The normal range for whole *blood creatine* is from 2 to 7 mg. per 100 ml. Whilst most workers have reported creatinine to be equally distributed between cells and plasma, at least in proportion to the amount of water in them, most of the creatine is found in the cells, there being only a small amount in the plasma, usually well under 1 mg. per 100 ml. Tierney and Peters report 0·2 to 0·6 mg. per 100 ml. in men, 0·35 to 0·9 mg. per 100 ml. in women. Figures of several mg. per cent. have been reported in the cells.

The plasma creatinine increases in renal disease. It appears that the proportion which is creatinine of the substances measured by the Jaffé reaction is smaller in nephritis than in health. As we saw earlier, creatinine increases later in renal failure than does urea, so that a value of over 5 mg. per 100 ml. in chronic nephritis is usually regarded as indicating a grave prognosis. Several workers have reported that the pre-renal factors which increase blood urea have little influence on the blood creatinine.

The evidence regarding the blood creatine in nephritis is conflicting. Its estimation has been comparatively little used. Serum creatine was shown to be increased in hyperthyroidism by Tierney and Peters (1943). Griffith (1951) has compared the serum creatine with the basal metabolism in this condition. A raised creatine, to between 0·6 and 1·6 mg. per 100 ml., may or may not be accompanied by increased basal metabolism. He considers that a normal serum creatine and normal basal metabolism excludes thyroid dysfunction, and holds that when symptoms of thyroid disease are present, a raised serum creatine is highly significant even although the basal metabolism is normal.

Creatinine clearance under certain conditions has been held to give a measure of the glomerular filtration rate and so to be equal to the inulin clearance. This appears to be reliable in health but may not be so when renal disease is present.

For a comprehensive review of the literature on creatine and creatinine see Peters and Van Slyke, 1946.

## URIC ACID

Most methods used for estimating urie aeid are based on the reaction with a phosphotungstic aeid reagent first used by Folin and Denis (1912–13). A very large amount of work has been done on this estimation and numerous modifications of the original and subsequent techniques have been published.

### Determination of Uric Acid in Blood.

#### I. Method of Brown (1945)

**Reagents.** 1. Sodium tungstate, 10 per eent. solution of $Na_2WO_4, 2H_2O$.

2. Sulphurie aeid, 2/3 N solution.

3. Urie acid reagent. Dissolve, with the aid of heat, 100 grams of sodium tungstate (analar $Na_2WO_4, 2H_2O$) and 20 grams of anhydrous disodium hydrogen phosphate in about 150 ml. of water. Mix 25 ml. concentrated sulphuric acid with about 75 ml. of water and pour the warm solution slowly into the flask whilst shaking continuously. Boil gently for one hour under a reflux eondenser. Brown suggested using as eondenser a funnel holding a 200 ml. conical flask filled with ice water. Cool under running water and make to 1 litre with water. The solution may have a slightly greenish yellow colour. It is not neeessary to decolorize with bromine.

4. Sodium eyanide, 12 per cent. solution. Keep in the refrigerator where the solution is stable for two weeks. It is necessary to use a high grade sodium eyanide or appreciable blanks may be obtained. Both the blank given by the cyanide solution and its keeping qualities should be studied.

5. Urea, 50 per cent. solution. Dissolve 50 grams of urea in water and make up to 100 ml. This solution keeps well at room temperature.

6. Stock standard uric acid solution. Weigh out 100 mg. of uric acid in a small beaker. Dissolve 60 mg. of lithium carbonate in 15 to 20 ml. of water in a test tube. Heat the solution to about 60° C. and pour on to the uric acid. Stir until dissolved, heating further in warm water if necessary. When dissolved, transfer with washing to a 100 ml. flask. Add 2 ml. of 40 per cent. formalin and then, slowly with shaking, add 10 ml. of 50 per cent. v/v acetic acid. Make to the mark with water and mix. Kept in a well-stoppered bottle away from the light, this solution will keep at least a year.

7. Standard solution for use. Dilute 1 ml. of the stock standard to 250 ml. This solution contains 0·004 mg. uric acid per ml.

**Technique.** Prepare the usual tungstic acid filtrate, and to 2 ml. (= 0·2 ml. blood) in a 10 ml. stoppered measuring cylinder, add 2 ml. of 12 per cent. sodium cyanide FROM A BURETTE, then 2 ml. of 50 per cent. urea, and finally 1 ml. of the phosphotungstic acid reagent, mixing after each addition. Stand for one hour in a bath at 25° C. Make up to 10 ml. and mix. Set up a standard by treating 2 ml. of the weak standard above, in the same way, and a blank using 2 ml.

of water. Compare in the colorimeter. With the photoelectric colorimeter, Brown uses a green filter, or transmission at 520 millimicrons. If a red filter is used, take 1 ml. of tungstic acid filtrate and 1 ml. of standard. The calculation is then the same except that for preparing a standard curve the stock standard must be diluted 1 in 200.

**Calculation.** Since the filtrate used is equivalent to 0·2 ml. of blood, and 2 ml. of the standard contain 0·008 mg. of uric acid, for the photoelectric colorimeter :

Mg. uric acid per 100 ml.

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}008 \times \frac{100}{0{\cdot}2}.$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 4.$$

A standard curve can be prepared as follows, using as standard a solution prepared by diluting the stock standard 1 in 100 and so containing 0·01 mg. per ml.

| | | | | | | |
|---|---|---|---|---|---|---|
| Mg. uric acid per 100 ml. blood . . . | 0 | 2 | 4 | 6 | 8 | 10 |
| Ml. standard uric acid solution (1 ml. = 0·01 mg.) | 0 | 0·4 | 0·8 | 1·2 | 1·6 | 2·0 |
| Ml. distilled water . . . . . | 2·0 | 1·6 | 1·2 | 0·8 | 0·4 | 0 |

Develop the colour as described above.

A blank should always be prepared, and with satisfactory reagents should give only a faint colour.

If the visual colorimeter is used, two standards containing 0·006 and 0·016 mg. of uric acid in 2 ml. of solution are suitable and cover almost all values found. These correspond to 3 and 8 mg. uric acid per 100 ml. respectively. If only one standard is put up the 4 mg. standard given above is most convenient.

**Notes.** Two important sources of error have been found in uric acid estimations. Some uric acid is carried down with the protein precipitate, giving low results, and there are present in blood other substances which also give a colour, tending to give high results. Such substances are mostly found in the red blood cells, so that error due to them can be largely avoided by using plasma or serum. Use of serum is recommended. Furthermore, should it be necessary to add additional sulphuric acid to complete the precipitation of the proteins, this influences the amount of colour developed. Such addition is more likely to be needed with whole blood than with serum.

King (1951) places whole blood into isotonic sodium sulphate so that the substances in the cell walls which give a colour are carried down when the protein is precipitated. King precipitates the proteins by adding the phosphotungstic acid reagent and then develops the colour by adding a cyanide-urea reagent to the supernatant fluid obtained on centrifuging. The method is adapted to use 0·2 ml. of whole blood.

Folin (1933, 1934) precipitated the uric acid from the protein-free filtrate as silver urate, from which it was set free and estimated colorimetrically. This eliminates the effect of interfering substances. An enzyme uricase is also known, which can remove uric acid from blood. Estimations done before and after its action have been used to give the uric acid present (Buchanan, Block, and Christman, 1945, Johnstone, 1952).

Recovery of added uric acid to tungstic acid filtrates has never been 100 per cent., and has sometimes been much lower. The above method is claimed to give a 95 per cent. recovery with a minimum interference from other reacting substances.

Molybdate-free sodium tungstate should be used. If necessary this can be prepared as follows (Folin, 1934). Dissolve 250 grams of sodium tungstate in 500 ml. water and make neutral to litmus with 5 N hydrochloric acid. Saturate with hydrogen sulphide and stand twenty-four hours. Add 400 ml. absolute alcohol gradually, with constant shaking. Filter after again standing overnight, wash the precipitate with 50 per cent. alcohol, and dissolve in 375 ml. of water. Add 0·5 ml. bromine and boil gently to remove excess of bromine. Make the hot solution alkaline to phenolphthalein by adding 40 per cent. sodium hydroxide. Cool, filter if necessary, add 200 ml. of absolute alcohol, and stand overnight. Filter off the white crystals and dry in a desiccator. However, high grade analytical reagent is almost always satisfactory without further purification.

### II. Method of Bidmead (Bidmead, 1951)

Bidmead has also developed a simple method using Benedict's arseno-phosphotungstate reagent.

**Reagents.** 1. Sodium tungstate, 10 per cent. solution of $Na_2WO_4$, $2H_2O$.

2. Sulphuric acid, 2/3 N solution.

3. Urea-cyanide reagent. Dissolve 25 grams of pure sodium cyanide in 400 ml. distilled water and add 75 grams of urea. Make up to 500 ml. Do not keep for more than two months.

4. Arseno-phosphotungstic reagent (Benedict, and Franke 1922). Dissolve 50 grams of molybdate-free sodium tungstate in 300 ml. of distilled water, followed by 25 grams of arsenic pentoxide. Then add 12·5 ml. of syrupy phosphoric acid and 10 ml. of concentrated hydrochloric acid. Boil for twenty minutes, cool, and make up to 500 ml. with water. This reagent keeps indefinitely.

5. Stock standard solution. Prepare as for the previous method.

6. Standard for use. Dilute 1 ml. of stock solution to 100 ml. with water.

**Technique.** Prepare a protein-free filtrate by the usual Folin technique. As above, use of serum is recommended. Take care to avoid hæmolysis. Measure 5 ml. of this into a centrifuge tube graduated at 10 ml., add 2 ml. of urea-cyanide solution, 0·2 ml. of arsenophosphotungstate reagent and make up to 10 ml. with water. Mix immediately, and stand for five minutes at room temperature. Then read in the colorimeter using a red filter, or transmission at

680 millimicrons, with the photoelectric colorimeter. Put up a blank using 5 ml. water, 2 ml. cyanide-urea solution, and 0·2 ml. of reagent and making up to 10 ml. as before. This should be clear and almost colourless. A standard curve can be prepared as follows :

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Mg. uric acid per 100 ml. serum . . . | 0 | 2 | 4 | 6 | 8 | 10 |
| Ml. standard uric acid (0·01 mg. per ml.) . | 0 | 1 | 2 | 3 | 4 | 5 |
| Ml. distilled water . . . . . | 5 | 4 | 3 | 2 | 1 | 0 |

Proceed as described for the test.

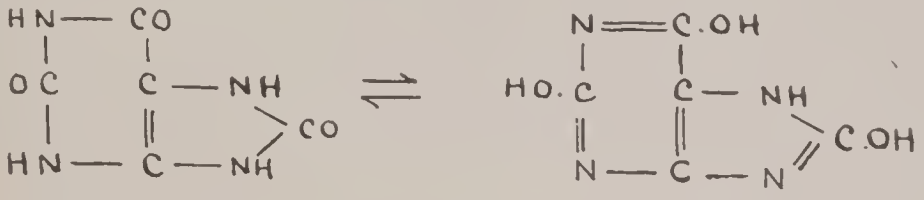
Put up the 4 mg. per 100 ml. standard with each batch of determinations.

For higher values use 2·5 ml. of protein-free filtrate.

**Note.** The temperature used for the colour development should be fixed within narrow limits. 20° C. is satisfactory. At lower temperatures the rate of colour development falls off appreciably.

INTERPRETATION

Uric acid belongs to, and is the end product of the metabolism of the group of substances known as the purines, some of which are present in nucleic acid found in the molecule of the nucleoproteins. It is accordingly formed endogenously from nucleoprotein metabolism, and exogenously from the metabolism of purines taken in the food. The formula of uric acid is given, and the type of isomerism possible is shown. From the formula it can be seen why uric acid forms salts, though it is only dibasic and not tribasic as might appear possible from the formula. Monosodium and disodium salts, for example, are known.




Keto-form ⇌ Enol-form

Uric Acid

There is some variation in the figures given for the normal range of blood uric acid. In part this may be due to the number of techniques used for its determination, and also to variations in the distribution between cells and plasma. Archer and Rhoden (1951) found the mean for whole blood to be 3·52 mg. per 100 ml., that for plasma, 4·04. In the majority of normal persons uric acid is between 2 and 5 mg. per 100 ml. However, values down to and very occasionally below 1·0 are obtained. As regards the upper limits of normal, a point of some importance in the use of uric acid determinations in gout, Steeher, Hersh and Solomon (1949), in a familial study of gout,

have placed this as high as 6·5 mg. per 100 ml. Bulger and Johns (1941) give 2 to 6 mg. per 100 ml. as the normal range, with a rather higher average for men than for women. Most other workers have given a lower maximum, from 3·5 to 5·0 mg. per cent. A small increase towards the end of pregnancy has been reported.

In disease the main interest centres on the findings in gout. In this, urates are deposited in the solid form in and about the joints and the blood level is often raised. All workers who have studied the blood uric acid in gout have reported a proportion within normal limits and have found that increases are not necessarily related to the severity of the attack. The range commonly seen in gout is from 5 to 10 mg. per 100 ml., though even higher figures have been reported. The determination may have some diagnostic value in differentiating gouty from non-gouty arthritis. It should be borne in mind that an increase in blood uric acid occurs when there is impaired renal function, and in conditions generally in which the blood urea is raised, when it may range from 4 to 20 mg. per cent. If the estimation is used for diagnostic purposes in connection with gout the presence of such a renal defect has to be ruled out. Though renal complications may well develop ultimately in chronic gout, apart from this, no increase in blood urea should be found to accompany the rise in uric acid in that condition.

Increases up to 10 mg. per 100 ml. have also been found in leukæmia. This appears to be due to the considerable breakdown of cell nuclei, with resulting increased metabolism of nucleoprotein. A similar explanation may also account for other increases in uric acid. An increase is quite frequently found in toxæmia of pregnancy and here also it is not related to an increase in blood urea. Some rise is sometimes seen in broncho and lobar pneumonia, in polycythæmia, and increase has also been noted in the remissions of pernicious anæmia and in the early stages of the response to liver treatment. In rheumatic conditions, in which uric acid is normal, the effect of cortisone is to tend to produce an increase in blood uric acid.

Uric acid estimations give little information of value to the clinician except in cases of gout.

### Determination of Uric Acid in Urine

Methods similar to those used for blood have been applied to urine. With direct methods using either the Folin phosphotungstic acid reagent or Benedict's arsenophosphotungstic acid reagent (p. 150) substances other than uric acid also contribute to the colour. As a result only 80 to 90 per cent. of the colour is ordinarily due to uric acid, and sometimes this may be lower. Thus direct methods are rather inaccurate but may be good enough for some purposes. To achieve greater specificity, separation as the silver salt (Folin, 1933, 1934) or estimation before and after the action of uricase (Buchanan *et al.*, 1945) is necessary.

In applying the direct methods the urine must be diluted, 1 in 100 often being convenient for twenty-four-hour specimens. The

method can then be carried out as described for the tungstic acid filtrate.

Keep the urine in the refrigerator or use a preservative such as thymol. Proceed with the determination immediately after diluting the urine.

**Calculation.** With Brown's method using 2 ml. of standard containing 0·008 mg. of uric acid, and 2 ml. of urine diluted 1 in 100.

Mg. uric acid per 100 ml. urine

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}008 \times \frac{100}{0{\cdot}02}.$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 40.$$

The standard curve given for the photoelectric colorimeter can be used, allowing for the different dilution, which is 1 in 10 in the case of blood.

With Bidmead's method, taking 5 ml. of urine diluted 1 in 100, and proceeding as described for blood filtrate, the given standards correspond to 0, 20, 40, 60, 80, 100 mg. uric acid per 100 ml. urine.

INTERPRETATION

The urine uric acid is made up of an exogenous part, the amount of which depends on the quantity of purine-rich foods in the diet, and of an endogenous part resulting from the breakdown of nucleoprotein. On a purine-free diet this latter is found to range from 0·3 to 0·5 gram daily. The amount excreted when purine-containing foods are being taken is higher, a typical figure on an average diet being 0·6 to 0·7 gram. On a high purine diet excretion of uric acid may exceed 1·0 gram daily.

Urine uric acid is rarely determined. It follows a characteristic course in gout, being low before an attack and increasing during the attack. A consistently high excretion is found in leukæmia and in conditions in which there is increased metabolism of nucleoprotein without impairment of renal function. Administration of cortisone and the adrenocorticotrophic hormone of the pituitary increases the excretion of uric acid. Cincophen has a similar effect.

### Urate Crystals in Material from Joints

Material removed from joints in gout often contains masses of needle-shaped crystals of sodium acid urate, which present a typical appearance microscopically. The murexide test (p. 482) can be used to confirm the presence of urates in this material.

## AMINO ACID NITROGEN

### Determination of Amino Acid Nitrogen in Blood

#### Method of Folin

(Frame, Russell and Wilhelmi, 1943 ; Russell, 1944). This method uses the brown colour produced when amino acids react with $\beta$-naphthoquinone 4-sulphonate in alkaline solution.

**Reagents.** 1. Sodium β-naphthoquinone–4–sulphonate, 0·5 per cent. solution in water. Prepare freshly within an hour before use.

2. Borax, sodium tetraborate, $Na_2B_4O_7$, 10 $H_2O$, 2 per cent. solution.

3. N/10 sodium hydroxide.

4. Phenolphthalein, 0·25 per cent. solution in alcohol.

5. Acid formaldehyde reagent, 0·3 N hydrochloric acid containing 3 ml. of 40 per cent. formaldehyde per litre.

6. N/20 sodium thiosulphate.

7. Stock amino acid solution, which contains 0·2 mg. amino acid nitrogen per ml. in 0·2 per cent. sodium benzoate dissolved in 0·7 N hydrochloric acid. Danielson (1933) recommends an equimolar mixture of glycine and glutamic acid, the colour from which more closely matches in shade that given by the test. To prepare this standard, weigh 53·6 mg. of glycine and 105 mg. of glutamic acid and dissolve in 0·2 per cent. sodium benzoate in 0·7 N hydrochloric acid, making up to 100 ml. with the same solution. If glycine only is used, prepare a solution in the 0·2 per cent. benzoate in hydrochloric acid containing 107·2 mg. of glycine per 100 ml.

8. Standard for use. Dilute 3 ml. of the stock standard to 100 ml. with 0·2 per cent. benzoate in acid. One ml. of this contains 6 μg. of amino acid nitrogen.

**Technique.** The ordinary Folin tungstic acid filtrate can be used, prepared from either whole blood or plasma. Pipette 5 ml. of this into a suitable test tube.(6 × 1 inch), add a drop of phenolphthalein and then N/10 sodium hydroxide, drop by drop, until a permanent pink colour is produced. Treat similarly 5 ml. of the amino acid standard (containing 0·030 mg. amino acid nitrogen), and set up a blank using water instead of blood or plasma to check that the sodium tungstate and the acid are ammonia free. If necessary add a small amount of water to get the same volume in all the tubes. Then add 1 ml. of the borax solution to each tube. Mix, add 1 ml. of freshly prepared naphthoquinone solution, mix again and place the tubes in a bath of briskly boiling water for ten minutes. Then remove and place in cold water for five minutes. Dilute almost to 13 ml. with water. To acidify the mixture and decolorize the excess naphthoquinone, add 1 ml. of acid formaldehyde reagent, mix, and add 1 ml. of N/20 thiosulphate. Make up to 15 ml. with water, mix, stand for ten to thirty minutes and read in the colorimeter, using a blue filter or transmission at 470 to 480 millimicrons.

**Calculation.** For the photoelectric colorimeter

Mg. amino acid nitrogen per 100 ml. of blood or plasma

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}030 \times \frac{100}{0{\cdot}5}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 6.$$

A suitable standard curve can be prepared as follows, using a solution containing 20 μg. amino acid nitrogen per ml., obtained by diluting 10 ml. of the stock standard to 100 ml.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Mg. amino acid nitrogen per 100 ml. | 0 | 2·0 | 4·0 | 6·0 | 8·0 | 10·0 | 12·0 |
| Ml. standard solution (1 ml.=20 μg.) | 0 | 0·5 | 1·0 | 1·5 | 2·0 | 2·5 | 3·0 |
| Ml. distilled water | 5 | 4·5 | 4·0 | 3·5 | 3·0 | 2·5 | 2·0 |

Treat as described for the standard above.

**Notes.** 1. The reaction should be carried out at *p*H 9·2 to 9·4, or less colour will be developed. If a drop or two excess N/10 sodium hydroxide is added during neutralization, colour development is not affected but low results may be obtained if there is incomplete neutralization to only a faint pink or transient pink with phenolphthalein. Since the volume of the sample should not exceed 5 ml. by more than a few tenths of a ml., if the test solution is strongly acid use more concentrated alkali first and then neutralize finally with the N/10 hydroxide.

2. Of naturally occurring substances ammonia and uric acid may lead to erroneous results, but these are not usually present in blood in amounts likely to introduce serious errors. One mg. per 100 ml. of uric acid gives a colour similar to that given by 0·1 mg. per 100 ml. of amino acid nitrogen. A colour may also be given by sulphonamides. A level of free sulphonamides of 10 mg. per 100 ml. in blood was found by Frame to be equivalent to about 0·8 mg. amino nitrogen per 100 ml.

**Other Methods.** For the ninhydrin method, in which carbon dioxide is produced by the action of ninhydrin on α amino groups, see Hamilton and Van Slyke, 1943 ; and Cramer and Winnick, 1943. The formol titration method has also been applied to blood (Van Slyke and Kirk, 1933).

INTERPRETATION

Rather different values for the blood amino-acid nitrogen are given by the different methods. ' There is agreement that the amount of amino-acids in the cells is appreciably higher than that in the plasma. The lowest normal range is given by the ninhydrin method. With this the range for plasma amino-acids has been mostly given as from about 3·4 to 5·4 mg. per 100 ml. Kirk with the formol titration found rather similar values to those reported for Folin's colorimetric method with a plasma range of 4·3 to 7·7 compared with Schmidt's 4·8 to 7·8 with the Folin method. Amino-acids in the cells are approximately twice as great. Macfadyan has stated that some amino-acids are liberated when blood clots so that the figures are a little higher for serum than for plasma.

Deamination of amino-acids occurs in the liver. This, however, is only impaired when there is severe liver damage. An increased level of amino-acid nitrogen from this cause is found especially in acute yellow atrophy. In this condition values from 15 to 25 mg. per 100 ml. of plasma are common. Dent records a case with 37 mg. per cent. and Rabinowitch one in which a value of 200 was observed. It should be noted that in acute infective and toxic hepatitis, and in chronic hepatitis, essentially normal findings are the rule. A small increase has been found in some cases of eclampsia.

Tests of liver function have been suggested in which amino-acids have been given both orally and intravenously and blood and urine amino acids studied afterwards. These have not gained acceptance as being of value in the diagnosis of liver disease.

Increased blood levels have been noted in advanced renal failure, and an increase of a few mg. per 100 ml. has been reported in leukæmia. Slight increases have been noted occasionally in a number of conditions.

A decrease in plasma amino acids has been reported to follow the injection of insulin.

**Determination of Amino Acids in Urine**

The colorimetric method using naphthoquinone sulphonic acid cannot be used satisfactorily with urine. The formol titration method has probably been most used though it is not so accurate as the ninhydrin method. A simpler, though less reliable method, is the copper titration method of Albanese and Irby.

1. **Formol Titration Method** (Kirk, 1936 ; Van Slyke and Kirk, 1933)

Amino acids contain both a basic and an acidic group. Formaldehyde destroys the basicity of the amino group and permits titration of the acid group. The *p*H is first adjusted to between 6 and 7, neutralized formaldehyde is added, and the mixture then titrated with sodium hydroxide to *p*H 9, using phenolphthalein as indicator. Ammonia also reacts with formaldehyde and must be removed, or be determined so that a correction can be made. Phosphates and any albumin present must also be removed.

**Reagents.** 1. Acetic acid, 10 per cent. (v/v).

2. N hydrochloric acid.

3. N sodium hydroxide (carbonate free).

4. Solid barium chloride.

5. Saturated solution of barium hydroxide.

6. Disodium hydrogen phosphate, 0·05 M solution.

7. N/100 sodium hydroxide, prepared by diluting the normal solution.

8. Neutral formaldehyde solution. Add the phenolphthalein to 36 to 40 per cent. formaldehyde in the proportion 0·2 ml. per 10 ml. and add N/10 sodium hydroxide until a faint pink colour is produced.

9. Carbon dioxide free water.

10. Neutral red, 0·1 per cent. solution in 50 per cent. alcohol.

11. Phenolphthalein, 0·1 per cent. solution in alcohol.

12. Phenolphthalein, 0·2 per cent. solution in alcohol.

13. Octyl alcohol.

14. Litmus paper.

**Technique.** Remove any protein present by adding 0·5 ml. of 10 per cent. acetic acid to 25 ml. of urine and heating on a steam bath for five to ten minutes. Wash into a 50 ml. volumetric flask or stoppered measuring cylinder. If protein is absent, measure 25 ml. of urine directly into the volumetric flask. For precipitation of

phosphate and carbonate, add 1 gram of barium chloride and a drop of octyl alcohol. Shake to dissolve the barium chloride and make alkaline to litmus paper with saturated barium hydroxide. Add a further 2·5 ml. of barium hydroxide and make to the mark with water. Stand for fifteen minutes and filter.

To remove ammonia proceed as follows : Pipette 25 ml. of the filtrate into the flask of a vacuum distillation apparatus. Add a few drops of octyl alcohol and a piece of litmus paper. Distil *in vacuo* for five minutes. Without interrupting the distillation, add N hydrochloric acid in 0·5 ml. portions until the litmus becomes red. Continue distilling for a further two minutes to remove carbon dioxide. Transfer to a 25 ml. stoppered glass cylinder, washing out several times with carbon dioxide-free water. Add 12 drops of neutral red indicator and neutralize with N sodium hydroxide. Make to the mark with carbon dioxide-free water.

Prepare standards as follows :

(*a*) *Neutral Standard.* Pipette 1 ml. of untreated urine into a test tube and add 1 ml. water, 0·4 ml. of 0·05 M disodium hydrogen phosphate, and 1 drop of neutral red indicator. Titrate with either alkali or acid to the point at which the colour changes sharply. The original urine must be used otherwise phosphate is precipitated.

(*b*) *Alkaline Standard.* Pipette 2 ml. of the urine filtrate into a test tube and add 1 drop of 0·1 per cent. phenolphthalein, and add 0·4 ml. of formaldehyde. Run in N/100 sodium hydroxide until the maximum colour is produced.

Titrate the urine filtrate as follows. Pipette 2 ml. into a test tube and add acid or alkali until the colour of the sample matches that of the neutral red standard. Add 0·4 ml. formaldehyde and 3 drops of 0·2 per cent. phenolphthalein, and titrate with N/100 sodium hydroxide until it matches the alkaline standard. The colour changes are from red to yellow to red. Note the ml. of N/100 alkali required in this titration. Carry out a blank using water instead of the urine filtrate. Use a microburette both for adjusting the *p*H and in the final titration.

**Calculation.** Mg. amino acid nitrogen in the sample
= 0·14 × (Titration of unknown − Titration of blank).
Since the sample taken is equivalent to 1 ml. urine
Mg. amino acid nitrogen per 100 ml. = 14 × (Titration of unknown − Titration of blank). The error of the titration appears not to be greater than 0·02 ml. of N/100 alkali, which is equivalent to 0·28 mg. amino nitrogen per 100 ml. urine.

### 2. Copper Method of Albanese and Irby

Albanese and Irby (1944) have applied the copper method of Pope and Stevens (1939) to urine. A suspension of copper phosphate is added to urine. A copper amino acid compound is formed in solution. The remaining copper phosphate is filtered off, and the copper in solution determined iodometrically.

**Reagents.** 1. Cupric chloride solution. Dissolve 27·3 grams of cupric chloride, $CuCl_2$, $2H_2O$, in 1 litre of water.

2. Trisodium phosphate solution. Dissolve 64·5 grams in 1 litre of water.

3. Borate buffer. Dissolve 57·21 grams of sodium borate in about 1,500 ml. water, add 100 ml. N hydrochloric acid and make to 2 litres with water.

4. Copper phosphate suspension. Mix 1 volume of cupric chloride solution, 2 volumes of trisodium phosphate, and 2 volumes of borate buffer. This keeps for one week in the refrigerator.

5. Thymolphthalein indicator. Dissolve 0·25 gram of thymolphthalein in 50 ml. of 95 per cent. ethyl alcohol and make to 100 ml. with water.

6. N/100 sodium thiosulphate solution. Prepare freshly before use from stock normal solution.

7. Starch indicator. One per cent. in saturated sodium chloride.

8. Potassium iodide. Prepare a solution containing 1 gram per ml. as required.

9. Glacial acetic acid.

10. N sodium hydroxide.

**Technique.** Collect twenty-four-hour urine specimens in brown bottles containing 50 ml. of 15 per cent. v/v hydrochloric acid and 1 ml. 10 per cent. alcoholic thymol. Make up to 2 litres with water. Such a urine keeps for a week at room temperature. Measure 15 ml. into a 50 ml. volumetric flask (this should contain at least 1·0 mg. of amino nitrogen) and add 4 drops of thymolphthalein indicator and run in N sodium hydroxide until a faint green or blue colour is obtained. Then add 30 ml. of the copper phosphate suspension from a graduated cylinder and make to the 50 ml. mark with water. Mix thoroughly by repeated inversion, stand for five minutes, and filter through No. 5 Whatman paper into a conical flask. Take two 10 ml. aliquots, and determine their copper content. Acidify with 0·5 ml. glacial acetic acid and add 1 ml. of potassium iodide solution. Titrate with N/100 thiosulphate using a microburette and 6 drops of starch indicator for the end point. For a low titre, titrate the duplicate with N/200 thiosulphate.

**Calculation.** Since 1 ml. N/100 thiosulphate = 0·28 mg. of amino-acid nitrogen,

Mg. urinary amino-acid nitrogen per twenty-four-hour specimen

$$= \frac{\text{ml. N/100 thiosulphate required} \times 0{\cdot}28 \times 2{,}000}{3}.$$

$$= \frac{\text{ml. N/100 thiosulphate required} \times 560}{3}.$$

INTERPRETATION

The daily excretion of amino-acid nitrogen as measured by the formol method ranges from 100 to 400 mg., forming from 1 to 2 per cent. of the total urine nitrogen. The copper method gives higher results ranging from 200 to 700 mg. daily. Even the formol method gives results higher than those obtained by the ninhydrin method. The copper method has been shown by Milne to include citrates present in the urine.

The increase in plasma amino acids in severe liver damage such as acute yellow atrophy, leads also to a marked increase in the daily excretion of amino acids in the urine. The presence of crystals of leucine and tyrosine in the urine deposit in this condition has been referred to earlier (p. 97). In less severe cases of liver damage there is not, however, any such increase. Increased excretion of amino acids in liver disease indicates a very grave prognosis.

Recently increased interest has arisen in some rare diseases in which there is abnormal excretion of amino acids in urine. The recent work of Dent and Rose on cystinuria has been described earlier. Whereas this condition has been shown to be most likely due to a tubular defect, the disease phenylketonuria (phenylpyruvic oligophrenia) appears to be due to a metabolic defect, the patient being unable to convert phenylalanine into tyrosine. Tyrosine can be metabolized normally but phenylalanine is present in increased concentration in blood, cerebrospinal fluid and urine. The phenylalanine is mostly excreted in the urine as phenylpyruvic acid. The condition has been studied by Jervis (1947) and by Lindan (see Dent, 1949).

Dent (1947, 1949) has also studied the Fanconi syndrome in which there is also a marked increase in amino acid excretion. In one case the output ranged from 491 to 1,655 mg. daily, average about 1,050 mg. The percentage amino nitrogen of the total nitrogen was from 3·1 to 13·0. Dent suggests that this figure should always be determined when considering a case of increased amino acid excretion. He considers any value over three by the formol method to be abnormal. Dent has shown that in the Fanconi syndrome the increased excretion is due to a low renal threshold for amino acids. The blood level is normal. The evidence for this has been mainly obtained by paper chromatography. For a full description of the techniques used, see Dent, 1947, 1948, 1949, 1951, and Dent and Shilling, 1948. Individual variations in amino-acid distribution are found but are typical for each particular case. Renal glycosuria is also present.

An increased excretion of amino acids is also found in Wilson's disease, hepato-lenticular degeneration, in this case associated with an increased urinary excretion of copper.

For cystinuria see p. 96.

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*CHAPTER XI*

# THE PLASMA PROTEINS

URINE proteins have already been considered. Here we shall be concerned with the plasma proteins, which include albumin, globulins and fibrinogen. Before dealing with the methods used for estimating these we shall discuss the question of their fractionation.

## FRACTIONATION OF PLASMA PROTEINS

The earliest fractionations were made using half-saturation with ammonium sulphate or a 22 per cent. concentration of sodium sulphate. The object of these was to separate albumin, left in solution, from globulins, which were precipitated. If plasma was used, fibrinogen was included in the globulins; with serum the separation was into albumin and globulins. Fibrinogen was determined separately by adding calcium chloride to plasma, collecting the fibrin clot formed, and estimating its amount. Subsequently Campbell and Hanna (1937) used a concentration of 21 per cent. of sodium sulphite for precipitating the globulins. Most of the determinations done clinically up to the present have used one or other of these three means of separating the albumin from the globulins, with 22 per cent. sodium sulphate the most used.

Much work has also been done on further fractionation of the plasma proteins, more especially of the globulins. For a full discussion of this subject see Edsall (1947) where the large scale fractionation methods of Cohn and his co-workers are also discussed, and Marrack and Hoch (1949). Amongst the earliest work was that of Howe (1921), who used a series of concentrations of sodium sulphate to separate three globulin fractions :

| | |
|---|---|
| Euglobulin . | Precipitated by 13·5 per cent. sodium sulphate. |
| Pseudoglobulin I . | Precipitated along with euglobulin by 17·4 per cent. sodium sulphate. |
| Pseudoglobulin II . | Precipitated along with both the above by 21·5 per cent. sodium sulphate. |

These were based on a study of the effect of increasing the concentration of sodium sulphate. Using serum diluted 1 in 30 the concentrations of sodium sulphate employed were 14, 18, and 22·2 per cent. respectively. By difference it was thus possible to separate the globulin fraction into three components, euglobulin, and pseudoglobulins I and II. The term euglobulin has also been used for the protein precipitated by one-third saturation with ammonium sulphate, the pseudoglobulins being then precipitated between one-third and one-half saturation with ammonium sulphate.

For this Howe fractionation Gutman *et al.* (1941) give the following normal ranges :

| | Mean | Range |
|---|---|---|
| Albumin | 5·2 per cent. | 4·7 to 5·7 |
| Globulin : | | |
| Euglobulin | 0·2 ,, ,, | 0·2 to 0·4 |
| Pseudoglobulin I | 1·3 ,, ,, | 0·8 to 1·9 |
| Pseudoglobulin II | 0·5 ,, ,, | 0·2 to 0·8 |
| Total Globulins | 2·0 ,, ,, | 1·3 to 2·4 |
| Total Protein | 7·2 ,, ,, | 6·5 to 7·9 |

A considerable step forward was taken with the application of electrophoresis to the fractionation of these proteins. The different mobility of the various components in an electric field is measured. For the technical side, see Tiselius (1937, 1939), Longworth (1942, 1945), and Armstrong *et al.* (1947). As a result the globulins have been divided into fractions designated $\alpha$ (later further separated into $\alpha_1$ and $\alpha_2$), $\beta$, and $\gamma$ globulins. Some examples of the results obtained by electrophoresis are as follows :

TABLE XII

*Electrophoretic Fractionation of Plasma Proteins*

| | I | II | III | IV | V |
|---|---|---|---|---|---|
| Albumin | 3·77 | 4·04 | 4·23 | 4·1 | 3·88 |
| Globulins : $\alpha_1$ | — | 0·31 | 0·28 | 0·5 | 0·58 |
| $\alpha$ | 1·39 | 0·79 | 0·96 | 1·1 | 1·34 |
| $\alpha_2$ | — | 0·48 | 0·68 | 0·6 | 0·76 |
| $\beta$ | 1·04 | 0·81 | 0·89 | 0·9 | 1·01 |
| $\gamma$ | 1·09 | 0·74 | 0·93 | 1·0 | 1·05 |
| Total | 3·52 | 2·34 | 2·78 | 3·0 | 3·4 |
| Fibrinogen | — | 0·34 | — | — | |
| Total protein | 7·29 (serum) | 6·75 (plasma) | 7·01 (serum) | 7·1 (serum) | 7·28 (serum) |

I. Levin *et al.* (1950) ; II. Dole and Braun (1944) ; III. Stirling and Rieketts (1949) ; IV. Rafsky *et al.* (1950) ; V. Seibert *et al.* (1947).

Although there is a considerable difference between the figures of different authors there is general agreement that the albumin fraction estimated after precipitation of globulins with 22 per cent. sodium sulphate is higher than that as measured by electrophoresis and it is now generally accepted that the albumin separated by that concentration of sodium sulphate contains $\alpha$ globulin.

Much work has been done comparing the sodium sulphate fractions with those of electrophoretic analysis. Majoor (1947) from a study of protein solubilities over a wide range of concentrations of sodium sulphate, suggested the use of 26·8 grams of sodium sulphate per

100 ml. of aqueous solution for precipitating all the globulins. He further used 18·5 grams per 100 ml. to precipitate the γ-globulins, so that the difference between the two gave the α and β globulins.

Milne (1947) also used 26·8 per cent. sodium sulphate to precipitate the whole of the globulins. In addition he used 19·6 per cent. sodium sulphate and held that this precipitated the β-and γ-globulins. He thus obtained three fractions which he considered to be albumin, α-globulin, and the sum of the β-and γ-globulins.

Kibrick and Blonstein (1948) used 15·75 per cent., 19·90 per cent., and 27·2 per cent. sodium sulphate solutions, to precipitate respectively the γ-globulins, the β-and γ-globulins, and the whole of the globulins. They used 10 ml. of these solutions with 0·5 ml. of serum. at 37° C., giving final concentrations of 15, 19, and 26 per cent. sodium sulphate. This procedure separates the proteins into four fractions, albumin, α, β- and γ-globulins, which were claimed to correspond reasonably well with those obtained by electrophoresis.

Wolfson *et al.* (1948) used 28·0 per cent. sodium sulphite to precipitate the globulins, 23·0 per cent. sodium sulphate to precipitate the β- and γ-globulins, and an ammonium sulphate-sodium chloride solution (see below) to precipitate the γ-globulins. Details of their method are given later.

Martin and Morris (1949) also studied the precipitation of total globulin and concluded that the use of 26 per cent. sodium sulphate and the methyl alcohol method of Pillemer and Hutchinson (see below, p. 164) give results which agree with the separation by electrophoresis, but that precipitation with 21 per cent. sodium sulphite (Campbell and Hanna) does not. Martin, Morris and Smith (1950) made further studies with sodium sulphate, using 13, 15, 18 and 26 per cent. solutions. They concluded that at 13 per cent. a significant amount of γ-globulin remains in solution, and are of the opinion that 15 per cent. affords a reasonable separation of the γ-globulins and 26 per cent. of the total globulins.

It is probably too much to hope for a very close correlation between salt fractionation and that by electrophoresis. At present it appears that the closest agreement is obtained using approximately 15, 20 and 27 per cent. concentrations of sodium sulphate. The agreement, however, is better with normal than with abnormal sera, but since each of the fractions, and this is particularly so with the γ-globulins, is heterogeneous, very close correlation is not to be expected and the greater divergence in the case of pathologieal sera is not surprising.

### Precipitation of γ-Globulin with Ammonium Sulphate

Several workers have used ammonium sulphate to precipitate the γ-globulin fraction. Thus Wolfson *et al.* (1948) used a solution containing 193 grams of ammonium chloride and 40 grams of sodium chloride per litre (see p. 171).

De la Huerga and Popper (1950) used a slightly different reagent containing 189 grams of ammonium sulphate and 29·3 grams of sodium chloride per litre. They have used this as follows to determine the concentration of γ-globulin. Measure 0·10 ml. of serum into

a test tube by means of a micropipette and add 5 ml. of the reagent. Stand thirty minutes and invert the tube twice without shaking, and immediately read the turbidity in a photoelectric colorimeter with the reagent as blank and using a red filter or transmission at 650 millimicrons. They express the result in the units read from the standard curve used for the thymol turbidity test (pp. 244, 463). or convert these into grams per 100 ml. as follows :

grams $\gamma$-globulin per 100 ml. serum

$$= \frac{U}{5{\cdot}8} + 0{\cdot}02\ (20 - U),$$ where U = units from thymol turbidity standard curve.

Jager and Nickerson (1948) used 33 per cent. saturation with ammonium sulphate using a saturated solution adjusted to *p*H 7 with dilute ammonium hydroxide.

### Use of Methyl Alcohol to Precipitate the Globulins

Pillemer and Hutchinson (1945) showed that at 0° C. almost all the serum albumin remains in solution at a concentration of 42·5 per cent. methyl alcohol, in the *p*H range 6·7 to 6·9, and at ionic strength about 0·03, the globulins being almost quantitatively precipitated under these conditions.

**Reagents.** 1. Methyl alcohol. Add 607 ml. methyl alcohol to 393 ml. distilled water. Mix, cool to 0° C. and make to 1 litre with cold methyl alcohol.

2. Acetate buffer. Dilute 72 ml. of 1 M acetic acid and 12 ml. of M sodium hydroxide to 1 litre with water.

**Technique.** Maintain the serum and all reagents between 0° C. and 1° C. Pipette 2 ml. of fresh serum into a 15 ml. conical centrifuge tube and add 1 ml. of acetate buffer with stirring. Then add 7 ml. of cold methyl alcohol again whilst stirring. Stand for half an hour at 0° C. Centrifuge at 0° C. or filter through a 42 Whatman paper in an ice box. The filtration can be done at room temperature provided sufficient filtrate for analysis. about 3 ml.. is obtained within five to seven minutes after removing from the cold. Take the aliquot for analysis immediately owing to the volatility of the methyl alcohol. Using normal serum the amount of albumin nitrogen per ml. of filtrate varied from to 1·1 to 1·8 mg.

## DETERMINATION OF PLASMA PROTEINS

While Kjeldahl methods properly carried out are the most reliable, they are more time consuming than colorimetric methods and need more care in execution. Although the colorimetric methods may not be so sound theoretically, they can give results which are sufficiently accurate for clinical requirements. The Kjeldahl distillation method is the most reliable and should be used for very accurate work. If a colorimetric method is used it should be checked against a Kjeldahl method.

The value of the recent work on salt fractionation outlined earlier

in this chapter still needs establishing as regards ordinary routine investigations. Practically the whole of the figures in the literature are based on fractionation with 22·5 per cent. sodium sulphate, 21 per cent. sodium sulphite, or half saturation with ammonium sulphate, fractionations which are still widely used. The methods given below can, however, be used with the other salt concentrations suggested.

### I. Kjeldahl Nesslerization Method

**Reagents.** 1. Sodium chloride, 0·9 per cent. solution.

2. Calcium chloride, 2·5 per cent. solution. Dissolve 2·5 grams of the dry anhydrous salt in water and make up to 100 ml.

3. Solutions for the fractionation.

(*a*) For the ordinary Howe fractionation. Sodium sulphate, 22·2 per cent. solution. Dissolve 222 grams of anhydrous sodium sulphate with warming in 700 ml. of water. Transfer to a litre volumetric flask whilst still warm and make to the mark with water. Keep in an incubator at 37° C. A similar fractionation is given by 21 per cent. sodium sulphite. Dissolve 42 grams of the crystalline salt, $Na_2SO_3$, $7H_2O$, in water and make up to 100 ml.

(*b*) Other salt concentrations can be used. Thus for the fractionation suggested by Kibrick and Blonstein (p. 163) prepare 15·75, 19·9, and 27·2 per cent. sodium sulphate solutions. Keep the two stronger solutions in the incubator.

The technique given below is for the Howe Fractionation in which the dilution of the albumin-containing filtrate is 1 in 31. Kibrick and Blonstein, however, add 0·5 ml. of serum to 10 ml. of each of their sulphate solutions, thus obtaining 1 in 21 dilutions of albumin, albumin + α-globulin, and albumin + α- and β-globulins. The method can easily be adapted to use this fractionation.

4. Digestion mixture. Sulphuric acid together with some catalyst is usually used, the best probably being selenium dioxide and potassium persulphate. King *et al.* (1937) use the former. Their digestion mixture consists of 1 : 1 sulphuric acid and water containing 1 gram of selenium dioxide per 100 ml. Pour 50 ml. of concentrated sulphuric acid very slowly with constant stirring into 50 ml. of distilled water. Dissolve 1 gram of selenium dioxide in the mixture, cool, and make up to 100 ml. with distilled water.

Other workers have used 1 : 1 sulphuric acid and water and added potassium persulphate, either powdered or in solution, during the course of the digestion.

5. Nessler's reagent. Prepare as described under blood urea, p. 104.

6. Standard ammonium sulphate solutions. (*a*) Stock solution, containing 0·3772 grams per 100 ml. of solution.

(*b*) Solution for use. Dilute 5 ml. of stock solution to 100 ml. with distilled water. This solution contains 18·86 mg. per 100 ml. or 4 mg. of nitrogen per 100 ml., that is 0·04 mg. N per ml.

**Technique.** *Total proteins.* Dilute the plasma or serum 1 to

50 with 0·9 per cent. sodium chloride, for example, 0·5 to 25 ml., or 0·2 ml. to 10 ml. This will give albumin + globulin in the case of serum and fibrinogen as well in the case of plasma.

*Fibrinogen.* Mix 0·5 ml. of plasma, 14 ml. of distilled water, and 0·5 ml. of 2·5 per cent. calcium chloride solution in a small beaker and allow to stand in an incubator at 37° C. until a clot is formed. Thirty minutes is usually sufficient but it may be necessary to leave longer, even overnight, before it is concluded a clot is not forming. Insert a fine glass rod into the clot and by rotating it collect the clot on to it. Press the rod against the side of the beaker to squeeze out any solution and to compress the clot. Take care to pick up on the rod any small pieces of clot which may have become detached. Dry by pressing carefully against a piece of filter paper. Then transfer the clot to the tube in which the digestion is to be carried out.

When the prothrombin content of the plasma is low, clotting may not occur even though there is a normal plasma fibrinogen. Clotting can then be brought about as follows (Lempert (1952) ). To 0·5 ml. of plasma add 5 ml. of 0·9 per cent. saline, 0·25 ml. of saline extract of dried human brain (see p. 255), and finally, 0·5 ml. of 0·18 per cent. calcium chloride. A fibrin clot forms within a few minutes.

**Albumin.** Mix 0·5 ml. of serum or plasma and 15 ml. of 22·2 per cent. sodium sulphate solution in a suitable tube, cork, and place in the incubator at 37° C. for three hours. The precipitation with sodium sulphite, used in the same proportions, can be carried out at room temperature, only ten to fifteen minutes standing being required. Filter, in the incubator if sulphate is used, using a retentive filter paper such as Whatman 42 or 44. The filtrate contains the albumin. Kingsley (1940) adds 5 to 10 ml. of ether to the mixture, stoppers, shakes vigorously, and centrifuges. The globulin then forms a layer between the ether and the clear albumin solution, which can be removed by means of a pipette, the mouth of which is closed whilst inserting.

**Digestion.** Pipette 1 ml. (= 0·02 ml. of plasma or serum) of the diluted serum or plasma for total protein, and 1 ml. (= 0·0323 ml. of plasma or serum) of the albumin-containing-filtrate into 6 × 1 inch test tubes, and to these and to a similar tube containing the fibrin clot if it is desired to determine fibrinogen, add 1 ml. of the digestion mixture. Heat carefully using a microburner. Add a glass bead if bumping tends to occur. The mixture first darkens, later becoming colourless. It is necessary to continue heating for approximately ten minutes after this point is reached. Take great care in carrying out this digestion (see note 2 on p. 138). Cool and add water to 10 ml. Pipette off 2 ml. into a 10 ml. stoppered measuring cylinder. Make up to 7 ml. with water, or gum ghatti (p. 104) if trouble due to opalescence or turbidity is experienced, and then add 3 ml. of Nessler's reagent. Into another cylinder pipette 1 ml. of the standard ammonium sulphate solution (containing 0·04 mg. of nitrogen), make to 7 ml. with water or gum ghatti, and add 3 ml. of Nessler's reagent. Read in the colorimeter. For the photoelectric colorimeter use a violet filter, transmission 440 millimicrons. For this method it is

necessary to determine the non-protein nitrogen separately. Also, check the reagents by carrying out a reagent blank.

**Calculation.** Using the photoelectric colorimeter

Mg. total protein nitrogen per 100 ml.

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}04 \times \frac{100}{0{\cdot}004} - \text{mg. non-protein nitrogen per 100 ml.}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 1{,}000 - \text{mg. non-protein nitrogen per 100 ml.}$$

To convert to grams total protein per 100 ml. serum or plasma, multiply by 6·25 and divide by 1,000.

For albumin, since the filtrate used is equivalent to 0·0323 ml. of serum

Mg. albumin nitrogen per 100 ml. of serum or plasma

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}04 \times \frac{100}{0{\cdot}00646} - \text{mg. non-protein nitrogen per 100 ml.}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 619 - \text{mg. non-protein nitrogen per 100 ml.}$$

Again, to convert to grams albumin per 100 ml. multiply by 6·25 and divide by 1,000.

For fibrinogen since 0·5 ml. of plasma was used

Mg. fibrinogen nitrogen per 100 ml.

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}04 \times \frac{100}{0{\cdot}1}.$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 40.$$

So Mg. fibrinogen per 100 ml. plasma

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 40 \times 6{\cdot}25,$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 250.$$

Standard curves should be prepared to study the range over which Beer's law is obeyed.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Grams total protein N per 100 ml. | 0 | 0·20 | 0·40 | 0·60 | 0·80 | 1·00 | 1·20 | 1·40 | 1·60 |
| Grams albumin N per 100 ml. | 0 | 0·124 | 0·25 | 0·37 | 0·50 | 0·62 | 0·74 | 0·87 | 0·99 |
| Mg. fibrinogen per 100 ml. plasma | 0 | 50 | 100 | 150 | 200 | 250 | 300 | 350 | 400 |
| Ml. ammonium sulphate standard (1 ml. = 0·04 mg. N). | 0 | 0·2 | 0·4 | 0·6 | 0·8 | 1·0 | 1·2 | 1·4 | 1·6 |

and further as desired.

Add water or gum ghatti to 7 ml. and 3 ml. of Nessler's reagent.

*Note.* These include the non-protein nitrogen in the case of total protein and albumin.

*Correction for Non-protein Nitrogen.* The non-protein nitrogen in most cases is almost negligible compared with the protein nitrogen. If it is ignored when it is within normal limits the error introduced only amounts to about 0·10 to 0·15 gram per cent. in the protein figures. Some workers have deducted 0·15 gram per cent. where it is known there is no renal disease and that the blood urea is normal. If the blood urea is increased, however, a large error may be introduced. Thus a blood urea of 200 mg. per 100 ml. gives an error of 0·6 gram per cent. A correction must be made if the non-protein nitrogen is not known to be normal.

### II. Wu's Colorimetric Method (Wu, 1922; Greenberg, 1929; Greenberg and Mirolubova, 1936)

The proteins are determined colorimetrically by means of the Folin and Ciocalteu phenol reagent, which is held to react with the phenol group in the tyrosine present in the protein. A tyrosine solution is used as standard, the tyrosine equivalent of each protein being known from comparison with Kjeldahl determinations.

**Reagents.** 1. Sodium chloride, 0·9 per cent. solution.

2. Solutions for fractionation of proteins. For the ordinary fractionation either 22·2 per cent. sodium sulphate or saturated ammonium sulphate solution is used.

3. Sulphuric acid, 2/3 N.

4. Sodium tungstate, 10 per cent. solution of $Na_2WO_4$, $2H_2O$.

5. Sodium carbonate, 20 per cent. solution.

6. Phenol reagent of Folin and Ciocalteu. Prepare as described on p. 310. Use the undiluted reagent.

7. Tyrosine standard, 0·2 mg. tyrosine per ml. Dissolve 100 mg. tyrosine in N/10 hydrochloric acid and make to 500 ml. with the acid.

**Technique.** 1. *Total Protein.* Dilute 0·2 ml. of serum or plasma to 3 ml. with 0·9 per cent. sodium chloride.

2. *Albumin.* Mix 15 ml. of the 22·2 per cent. sodium sulphate solution and 0·5 ml. of serum in a test tube. Shake well, cork, and stand in an incubator at 37° C. for three hours. Filter through a No. 42 Whatman paper. If the filtrate is not clear at first, return to the same filter paper until a clear filtrate is obtained. As before 21 per cent. sodium sulphite solution may be used in the same way at room temperature. Globulins may also be precipitated by ammonium sulphate. Add 2 ml. of distilled water to 0·5 ml. of serum or plasma, mix, and add 2·5 ml. of saturated ammonium sulphate solution, mix and stand for at least an hour before filtering.

3. *Fibrinogen.* Add 1 ml. of plasma to 28 ml. of distilled water. Then add 1 ml. of 2·5 per cent. calcium chloride solution and collect the clot as described previously.

**Development of Colour.** (*a*) *Total Protein and Albumin.* Into two centrifuge tubes graduated at 10 ml., measure 0·4 ml. of the diluted serum and 0·4 ml. of the albumin-containing-filtrate if ammonium sulphate is used, 1·24 ml. for sodium sulphate or sulphite precipitation. Add 2 ml. of water and then 0·2 ml. of 10 per cent. sodium tungstate and 0·2 ml. of 2/3 N sulphuric acid. Mix well,

stand for a few minutes, and centrifuge. Remove the supernatant fluid, add about 5 ml. of water, and 2 drops of 20 per cent. sodium carbonate. Mix well to dissolve the precipitate. Then add 0·2 ml. of the Folin and Ciocalteu reagent, mix, and add 1·2 ml. of 20 per cent. sodium carbonate. Mix, make up to 10 ml., and place in a water bath at 37° C. for fifteen minutes. At the same time put up a standard. To 0·4 ml. of the tyrosine solution add about 5 ml. of water, 0·2 ml. of Folin and Ciocalteu reagent, and 1·2 ml. of 20 per cent. sodium carbonate. Make up to 10 ml. and develop the colour in the same way. Read in the colorimeter using a red filter or transmission at 680 millimicrons.

(*b*) *Fibrinogen.* Transfer the clot to a 25 ml. stoppered measuring cylinder, add a few ml. of water and 3 drops of 20 per cent. sodium carbonate solution. Mix well until the clot has dissolved. Add 0·5 ml. of the Folin and Ciocalteu reagent and 3 ml. of 20 per cent. sodium carbonate, and make up to 25 ml. with water. Develop the colour as before.

**Calculation.** The tyrosine equivalents of the plasma proteins are : 1 mg. tyrosine is equivalent to 27·5 mg. albumin, to 25·2 mg. globulin, and to 14·4 mg. of fibrinogen. For the photoelectric colorimeter we have

(*a*) *Total Protein.* Since 0·4 ml. of serum diluted 1 in 15, is used and the tyrosine standard contains 0·2 mg. per ml., that is 0·08 mg. per 0·4 ml. :

Mg. tyrosine equivalent to total protein per 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}08 \times \frac{15}{0{\cdot}4} \times 100$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 300.$$

For the usual albumin-globulin ratio the equivalent for total protein is very nearly 26·7 mg. per mg. tyrosine. Even at very high globulin levels the error due to using this is not more than about 4 per cent. and so is not clinically significant. This gives us

Grams total protein per 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}3 \times 26{\cdot}7 \text{ i.e. } \times 8.0$$

(*b*) *Albumin.* The volume of filtrate used is equivalent to 0·04 ml. serum. So

Mg. tyrosine equivalent to albumin per 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}08 \times \frac{100}{0{\cdot}04}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 200.$$

Hence : Grams albumin per 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}2 \times 27{\cdot}5 \text{ i.e. } \times 5{\cdot}5.$$

(*c*) *Globulin.* This is obtained by subtracting the mg. tyrosine equivalent to albumin per 100 ml. from the mg. tyrosine equivalent to total protein per 100 ml. and multiplying by 25·2, or with the small error referred to above, by subtracting the grams albumin per 100 ml. from the grams total protein per 100 ml. as calculated above.

(*d*) *Fibrinogen.* The volume of plasma equivalent to the tyrosine in the 10 ml. final volume of standard is 0·4 ml. Hence

Mg. fibrinogen per 100 ml. plasma

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}08 \times \frac{100}{0{\cdot}4} \times 14{\cdot}4$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 288.$$

A standard curve can be prepared as follows :

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Tyrosine equivalent to total protein per 100 ml. | 0 | 60 | 120 | 180 | 240 | 300 | 360 |
| Grams total protein per 100 ml., using factor 26·7 | 0 | 1·6 | 3·2 | 4·8 | 6·4 | 8·0 | 9·6 |
| Tyrosine equivalent to albumin per 100 ml. | 0 | 40 | 80 | 120 | 160 | 200 | 240 |
| Grams albumin per 100 ml., using factor 27·5 | 0 | 1·1 | 2·2 | 3·3 | 4·4 | 5·5 | 6·6 |
| Mg. fibrinogen per 100 ml. plasma | 0 | 58 | 115 | 173 | 230 | 288 | 346 |
| Ml. tyrosine standard (1 ml. = 0·2 mg.) | 0 | 0·2 | 0·4 | 0·6 | 0·8 | 1·0 | 1·2 |

Add to this, 10 ml. water, 0·5 ml. Folin Ciocalteu reagent, 3 ml. of 20 per cent. sodium carbonate solution, and make up to 25 ml. with water. Mix, and develop the colour as described for the test.

If only albumin and globulin are required it is better to use serum. If fibrinogen is needed as well, plasma must be used and the tyrosine equivalent of the fibrinogen subtracted before calculating the albumin and globulin. Fibrinogen is precipitated with the globulins.

This is a convenient and simple method, but is open to some objections. The amount of colour obtained from a given amount of protein tends to diminish on keeping the serum or plasma. It is advisable to make a practice of carrying out the determination during the twenty-four hours following taking the blood though little alteration would appear to occur for an appreciably longer period. It is important that the amounts of phenol reagent and alkali be adhered to, or the figures for tyrosine equivalence will not apply. Furthermore, this is obviously a highly empirical method. The figures for the tyrosine equivalents apply to normal albumin and globulin, and are not necessarily valid for abnormal proteins. Good agreement with the Kjeldahl method is obtained except when abnormal proteins are present such as those in multiple myeloma, but although there may be considerable divergence in these cases this does not affect the clinical significance since a marked increase in globulin is shown by both methods.

## III. The Biuret Method

This method uses the colour produced by treating diluted serum or plasma with a biuret reagent containing copper sulphate. This

is matched against standard protein solutions similarly treated. Many papers have been published on this method among which the following may be noted, Kingsley (1942), Mehl (1945), Weichselbaum (1946). Gornall *et al.* (1949). Wolfson *et al.* (1948) have applied the method to the fractionation of the serum globulins. They use 28 per cent. sodium sulphite (giving a final concentration of 26·88 per cent.) to precipitate all the globulins, 23 per cent. sodium sulphate (final concentration 21·16 per cent.) to precipitate the $\beta$ and $\gamma$ globulins, and an ammonium sulphate-sodium chloride solution to precipitate the $\gamma$ globulins. Fractionation can be carried out with the sodium sulphate solutions according to Kibrick and Blonstein (p. 165). The method can also be used with the original Howe fractionation using 22·2 per cent. sodium sulphate to separate the albumin from the globulin, or with the 21 per cent. sodium sulphite of Campbell and Hanna, but as stated above a considerable proportion of the $\alpha$-globulin will then be included with the albumin.

### Method of Wolfson, Cohn, Calvary, and Ichiba (1948)

**Reagents.** 1. Sodium sulphate, 23 per cent. solution. Dissolve 23 grams of the anhydrous salt in distilled water, make to 100 ml. and keep at 37° C.

2. Sodium sulphite, 28 per cent. solution. Dissolve 28 grams of the anhydrous salt in distilled water at 28° C., make to 100 ml. and keep at room temperature.

3. Sodium chloride-ammonium sulphate solution. Dissolve 193 grams of ammonium sulphate in about 500 ml. of distilled water, add 40 grams of sodium chloride, dissolve and make up to a litre. Keep at room temperature.

4. Biuret reagent (Weichselbaum, 1946). First dissolve 45 grams of Rochelle salt in about 200 ml. of N/5 sodium hydroxide. Then add 5 grams of copper sulphate ($CuSO_4$. $5H_2O$), and when this has dissolved add 5 grams of potassium iodide, and finally make to a litre with the N/5 sodium hydroxide. Keep in a waxed glass bottle with a rubber stopper.

5. Span ether reagent. Mix 1 ml. of Span 20 (Messrs. Honeywill and Stein, Ltd., 21 St. James's Square, London, S.W.1) and 99 ml. of ether. Filter and make up to 100 ml. again with ether. Keep tightly corked.

6. Standard protein solution. Take some pooled human serum and determine the protein content accurately by a Kjeldahl method. Use this to construct a standard curve as described under determination of total protein below.

**Technique.** 1. *Serum Total Protein.* Dilute 0·2 ml. of serum to 5 ml. with distilled water and mix well. To 3 ml. of this diluted serum add 3 ml. of biuret reagent, mix well, and stand for thirty minutes. Then read in the photoelectric colorimeter using a yellow-green filter or transmission at 540 millimicrons. As blank use a mixture of 3 ml. distilled water and 3 ml. of biuret reagent.

To prepare a standard curve dilute the pooled human serum of

known protein content so as to obtain a solution containing 400 mg. protein per 100 ml. Set up the following tubes :

| Grams protein per 100 ml. serum | 0 | 2 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|---|
| Ml. standard protein solution (400 mg. per 100 ml.) | 0 | 0·6 | 1·2 | 1·8 | 2·4 | 3·0 |
| Ml. distilled water | 3 | 2·4 | 1·8 | 1·2 | 0·6 | 0 |

Add 3 ml. biuret reagent to each and read as described above.

The result is then read directly from this curve.

2. *Serum Albumin plus α-Globulin.* Add 0·2 ml. serum to 2·3 ml. of 23 per cent. sodium sulphate. The authors recommend the use of a small tube graduated at 2·3 ml. for measuring out the sulphate since the salt tends to crystallize out when the solution is pipetted. Add to the mixture 1 ml. of ether, shake well, centrifuge, withdraw 1·5 ml. of the clear aqueous layer, add 1·5 ml. of distilled water and 3 ml. biuret reagent, mix, stand thirty minutes and read as for the total protein, using the same blank. The result is read from the same standard curve.

3. *Serum Albumin.* Add 0·2 ml. serum to 4·8 ml. of 28 per cent. sodium sulphite and mix thoroughly by inverting. Add 1 ml. of Span ether reagent and shake well for half a minute. Centrifuge and carefully withdraw 3 ml. of the aqueous phase and add to it 3 ml. of biuret reagent. Read after thirty minutes using as blank a mixture of 3 ml. of the sulphite solution and 3 ml. of biuret reagent. The result again is read directly from the above curve.

4. *Serum γ-Globulin.* Measure 9·6 ml. of the sodium chloride-ammonium sulphate solution into a 15 ml. centrifuge tube. Layer 0·4 ml. of serum on top and mix by slow, careful inversion until the turbidity appears to have reached a maximum. While still thoroughly mixed, remove 1 ml. and discard. Cork the tube and centrifuge for half an hour at about 2,500 r.p.m. If the supernatant fluid is still not quite clear, cool in running cold water and centrifuge again. For accurate results the supernatant fluid must be crystal clear. Carefully pour off most of the supernatant fluid without disturbing the precipitate, centrifuge again for a few minutes, carefully invert and stand on a filter paper to drain as completely as possible. Add 3 ml. of the biuret reagent and 3 ml. distilled water, and shake vigorously for half a minute. Stand fifteen minutes, centrifuge to clear, and take some of the supernatant fluid for reading in the colorimeter after it has stood not less than thirty minutes. Use as blank a mixture of 3 ml. of water and 3 ml. of biuret reagent. Read the grams protein per 100 ml. on the above standard curve and divide by three to obtain the concentration of γ globulin.

**Total globulin** is obtained by subtracting the albumin from the total protein and is used to calculate the true albumin-globulin ratio. *α-globulin* is obtained by subtracting the serum albumin from the serum albumin plus α-globulin. Serum β plus γ-globulin is obtained by subtracting the albumin plus α globulin from the total protein and the *β-globulin* obtained by subtracting the γ-globulin from this. In this way four fractions, albumin, α, β and γ-globulins are obtained.

IV. **Copper Sulphate Specific Gravity Method** (Phillips, Van Slyke, Dole, Emerson, Hamilton, Archibald 1945)

A graded series of copper sulphate solutions covering a convenient range of specific gravities is prepared and the specific gravity of whole blood, serum, or plasma, is ascertained by allowing drops to fall into these solutions under carefully controlled conditions and observing if they rise, fall, or remain suspended. From the specific gravity thus determined, the total protein content of serum or plasma can be read from previously prepared diagrams. By determining the specific gravity of both plasma and whole blood and doing hæmatocrits, values for hæmoglobin can be obtained.

On entering the copper sulphate solution the drop becomes surrounded with a layer of copper proteinate, remaining as a discrete drop without changing its gravity for fifteen to twenty seconds. During that time, whether it falls or rises shows whether its specific gravity is higher or lower than that of the solution. A constant size of drop is not necessary and no correction for temperature has to be applied since the coefficients of expansion of the copper sulphate solutions and of plasma and of blood are sufficiently near to each other.

For precise work, for plasma, twenty solutions are used covering the range of specific gravity 1·016 to 1·035. For whole blood forty solutions are used over the range 1·036 to 1·075.

### Preparation of the Copper Sulphate Solutions

Two methods have been used for preparing the standard copper sulphate solutions from which the series is made. In one, a saturated solution at a carefully measured temperature is prepared, and then diluted to give the standard used. In this case the quality of the copper sulphate used is not so important and no weighing is necessary. In the other method a carefully weighed amount of copper sulphate, exactly $CuSO_4$, $5H_2O$ in composition, is dissolved in water and made up exactly to a certain volume. Both these methods will be described.

(*a*) **Using a Saturated Solution.** Place about 4 lb. of crystalline copper sulphate (fine crystals or powder), fine enough to pass through a 20-mesh sieve, into a 4-litre bottle and add $2\frac{1}{2}$ litres of water at room temperature. Stopper and shake vigorously for five minutes. If there are signs of tiring pass the bottle to a second person to continue the shaking. At the end of five minutes put the bottle down, immediately insert a thermometer and read the temperature to the nearest 0·5° C. Then, leaving the bulk of the crystals in the bottle, filter at once, using an 18 to 20 cm. diameter funnel, keeping the funnel filled with solution. Proceed with the preparation of the stock solution at once. The volume of solution to be diluted to make 1 litre is read from Table XIII.

Measure the necessary amount into a litre flask using a 500 ml. cylinder and adding any excess accurately from a burette. Make up to the mark and mix. Stand. A little shrinkage in volume occurs,

TABLE XIII

*Ml. Saturated Copper Sulphate Solution to be Diluted to 1 litre to give the Stock Standard*

| °C. | 10·0 | 10·5 | 11·0 | 11·5 | 12·0 | 12·5 | 13·0 | 13·5 | 14·0 |
|---|---|---|---|---|---|---|---|---|---|
| Ml. of solution to dilute to 1 litre | 587 | 581 | 575 | 569 | 563 | 557 | 552 | 546 | 541 |
| °C. | 14·5 | 15·0 | 15·5 | 16·0 | 16·5 | 17·0 | 17·5 | 18·0 | 18·5 |
| Ml. of solution to dilute to 1 litre | 536 | 531 | 527 | 522 | 518 | 514 | 509 | 505 | 501 |
| °C. | 19·0 | 19·5 | 20·0 | 20·5 | 21·0 | 21·5 | 22·0 | 22·5 | 23·0 |
| Ml. of solution to dilute to 1 litre | 497 | 493 | 489 | 485 | 481 | 477 | 474 | 470 | 466 |
| °C. | 23·5 | 24·0 | 24·5 | 25·0 | 25·5 | 26·0 | 26·5 | 27·0 | 27·5 |
| Ml. of solution to dilute to 1 litre | 463 | 459 | 456 | 453 | 450 | 446 | 443 | 440 | 438 |
| °C. | 28·0 | 28·5 | 29·0 | 29·5 | 30·0 | 30·5 | 31·0 | 31·5 | 32·0 |
| Ml. of solution to dilute to 1 litre | 435 | 432 | 429 | 427 | 424 | 421 | 418 | 415 | 412 |

so after standing a minute make up to the mark again and mix. Transfer to a 4-litre bottle and prepare further solution until 4 litres have been obtained. The specific gravity should be 1·100.

The important points in the preparation of the saturated solution are that the copper sulphate should be fine enough, should be in sufficient excess, and that the temperature should be read immediately the five minutes shaking is ended. If the temperature is measured to the nearest 0·5° C., standards for plasma gravities accurate within ± 0·0002 can be prepared.

(*b*) **Accurate Weighing of the Copper Sulphate.** The stock copper sulphate solution of gravity $D_{25}^{25} = 1{\cdot}100$ can be prepared by

## Preparation of the Sets of Copper Sulphate Standards

For serum or plasma specific gravities dilute as follows :

| Specific Gravity | Ml. of solution of S.G. 1·100 to dilute to 100 ml. | Specific Gravity | Ml. of solution of S.G. 1·100 to dilute to 100 ml. |
|---|---|---|---|
| 1·015 | 14·26 | 1·026 | 25·12 |
| 1·016 | 15·25 | 1·027 | 26·10 |
| 1·017 | 16·24 | 1·028 | 27·08 |
| 1·018 | 17·23 | 1·029 | 28·06 |
| 1·019 | 18·22 | 1·030 | 29·04 |
| 1·020 | 19·21 | 1·031 | 30·00 |
| 1·021 | 20·20 | 1·032 | 31·00 |
| 1·022 | 21·19 | 1·033 | 32·00 |
| 1·023 | 22·17 | 1·034 | 33·00 |
| 1·024 | 23·15 | 1·035 | 34·00 |
| 1·025 | 24·14 | | |

accurately weighing the neeessary amount of copper sulphate. Either dissolve 159·0 grams of pure crystals in water and make up to a litre at 25° C., or mix a weighed amount of crystals with a measured volume of water providing 5·897 grams of water for each gram of crystals ; 170 grams to 1002·4 grams of water is convenient. The copper sulphate must have the exact composition, $CuSO_4$. $5H_2O$. On heating 2 or 3 grams in open Pyrex weighing bottles at 300° to 350° C. to constant weight (two to three hours), the loss in weight should be 36·6 $\pm$ 0·2 per cent. Crystals ean be reerystallized from hot water and dried at room temperature. Cheek the speeifie gravity of the solution by aeeurate weighing.

**Anticoagulants.** Heller and Paul's Oxalate Mixture. Dissolve 2 grams of potassium oxalate and 3 grams of ammonium oxalate in 250 ml. water. This is devised to have little effect on the cells. Use 2 mg. per ml. blood. Heparin may be used, 0·2 mg. per ml. blood, and is the more satisfaetory. Serum is preferred and should be used when this is possible.

**Technique.** In collecting the blood specimen do not apply a tourniquet for more than one minute.

Allow the serum or plasma to fall from a height of about 1 eentimetre above the solution. A syringe needle is suitable. If small drops are used the standard solution lasts longer. In delivering the drop steady the needle or pipette on the edge of the bottle. The drop passes some 2 to 3 em. below the surfaee. Within five seeonds the original momentum is lost and the drop is then seen to eontinue to fall, to beeome stationary, or to start to rise. Some fifteen to twenty seconds in all are available to observe its behaviour before the speeific gravity of the drop begins to ehange. It is thus the behaviour of the drop during the period five to fifteen seconds after its release which is crucial and must be observed earefully. If the relative rate of fall or rise of two conseeutive solutions is noted it is often possible to decide to which one the serum or plasma is nearer.

**Points to Note.** If a drop fails to pass eleanly through the surfaee film and remains attaehed to it by a tentaele, detaeh by shaking gently, and repeat. Even a minute air bubble attached to the drop of serum or plasma causes it to float and again it is neeessary to repeat.

Avoid conveetion eurrents in the standard solutions so allow these to adjust to the temperature of the room, and keep out of eurrents of air, hot or eold, and hold bottles by the top above the liquid.

Up to one-fortieth of its volume of serum or plasma (or blood) ean be added to a standard before its speeifie gravity is altered by 0·0005. The change is a deerease. One hundred ml. of standard should thus be sufficient for 100 tests if small drops are used.

**Calculation.** The total protein content of serum or plasma is calculated from the equation,

Plasma protein eoncentration in grams per cent.

$$= 360 \text{ (plasma S.G. } - \text{ 1·0070)}$$

1·0070 is the specific gravity of protein-free plasma ultrafiltrate.

Using this formula we get the following relation between specific gravity and total protein :

| S.G. plasma | Protein gm. per cent. | S.G. plasma | Protein gm. per cent. | S.G. plasma | Protein gm. per cent. |
|---|---|---|---|---|---|
| 1·015 | 2·88 | 1·022 | 5·40 | 1·029 | 7·92 |
| 1·016 | 3·24 | 1·023 | 5·76 | 1·030 | 8·38 |
| 1·017 | 3·60 | 1·024 | 6·12 | 1·031 | 8·64 |
| 1·018 | 3·96 | 1·025 | 6·48 | 1·032 | 9·00 |
| 1·019 | 4·32 | 1·026 | 6·84 | 1·033 | 9·36 |
| 1·020 | 4·68 | 1·027 | 7·20 | 1·034 | 9·72 |
| 1·021 | 5·04 | 1·028 | 7·56 | 1·035 | 10·08 |

It should be noted that other formulæ have been used. In earlier work 343 was used instead of 360, being obtained from observations on nephritic patients. Later a figure of 377 was obtained from normal patients by comparing with macro Kjeldahl estimation and a figure of 360 from miscellaneous hospital patients. This latter, also obtained by some other workers, was finally adopted. However, Hoch and Marrack (1945) have used 364 (S.G. plasma — 1·006) which gives values about 0·4 per cent. higher than the above.

The error in most types of normal and pathological cases varies from ± 0·3 gram per 100 ml. ; in severe nephrosis and nephritis, errors up to ± 0·6 gram per cent. may be found.

For hæmoglobin determinations a series of forty copper sulphate solutions are required with specific gravities from 1·036 to 1·075. They are prepared by diluting the stock solution of specific gravity 1·100 as follows : S.G. 1·036, 35 ml. to 100 ml. ; S.G. 1·037, 36 ml. to 100 ml. and so on. Line charts are available for reading off the hæmoglobin from plasma and whole blood specific gravities. For these see the original papers.

## Separation of Proteins by Paper Electrophoresis

A simple apparatus for qualitative and rough quantitative fractionation of plasma proteins has been described by Flynn and Mayo (1951), a diagram of which is shown in Fig. 36. While some laboratories will be able to construct their own, a commercial model has been produced by the Shandon Scientific Company, London (Fig. 37). This consists of a perspex tank covered with a heavy glass lid. The tank is divided into five compartments by four partitions which run the length of the tank at its base. A buffer solution, *p*H 8·6 (10·3 grams sodium diethylbarbiturate and 1·84 grams diethylbarbituric acid per litre) is placed in all compartments except the centre one. Carbon electrodes are in the two outer compartments. The filter paper strips dip into the two inner compartments which are connected electrically by a series of short wicks to the compartments containing the electrodes. This arrangement prevents *p*H changes at the electrodes from affecting the inner compartments. The filter paper strips are suspended over a stainless steel wire.

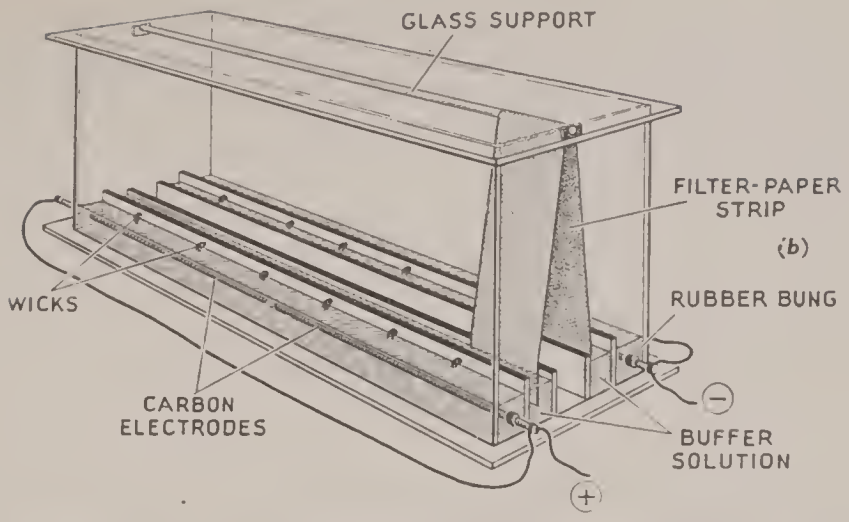


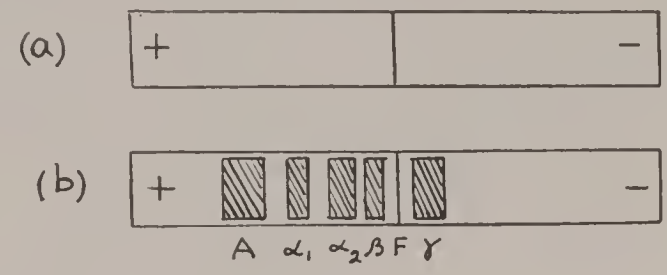

FIG. 36. Apparatus for Paper Electrophoresis (Flynn and Mayo). (*The Lancet.*)

FIG. 37. Apparatus for Paper Electrophoresis (Shandon Scientific Co.).

The tank stands on the box containing the electric power unit, which is mounted on levelling screws so as to keep the liquid at the same level in the four compartments. This unit takes A.C. from the mains and supplies D.C. up to 150 volts and 15 milliamps.

When in use, buffer solution is placed in the four compartments to the level of the wicks. Strips of Whatman No. 1 paper are placed over the steel wire. The glass lid is replaced and the apparatus left for about an hour for the paper to become saturated. Then 0·015 ml. of the serum or plasma is applied at the apex of the strip, and a potential of about 120 volts applied for about twenty hours. After carefully removing the strips they are placed horizontally in an oven at 105° C. for ten minutes to dry. They are then immersed in a staining reagent (a saturated solution of Naphthalene Black 12 B. 200 (I.C.I.) in methyl alcohol solution containing 10 per cent. acetic acid). After

draining they are washed free from excess dye in successive baths of fresh methyl alcohol—acetic acid. When the background is pale blue the strips are given a final washing in methyl alcohol only, to remove acetic acid, and dried in a current of warm air. A separation




POSITION OF PROTEINS (a) BEFORE AND (b) AFTER ELECTROPHORESIS :- A, ALBUMIN ; $\alpha_1$ $\alpha_2$ $\beta$ $\gamma$ CORRESPONDING GLOBULINS ; F FIBRINOGEN.

FIG. 38. Example of Paper Electrophosires.

of 10 to 12 cm. is obtained between the albumin and the $\gamma$-globulin as shown in Fig. 38.

PLASMA PROTEINS. INTERPRETATION

Total plasma proteins average just over 7 grams per cent., with a range from 6·3 to 7·9. The ranges for the main constituents are given a little differently by different workers but may be taken to be something like :

| | |
|---|---|
| Albumin . . | 3·6 to 5·8 per 100 ml. |
| Globulin . . | 1·4 to 3·1 per 100 ml. |
| Fibrinogen . | 200 to 400 mg. per 100 ml. |

giving an albumin-globulin ratio which varies from about 3 : 1 to about 1·5 : 1, most frequently being near to 2 : 1. Most figures given up to the present refer to fractionation using the Howe 22·2 per cent. sodium sulphate, half-saturation with ammonium sulphate, or 21·5 per cent. sodium sulphite. If the use of a higher concentration of sodium sulphate should become accepted it would seem that values for albumin will be rather lower, and those for globulin a little higher, than those given by methods used up to the present. The albumin-globulin ratio will also average a little lower, as is the case with electrophoretic fractionation.

In considering variations in plasma proteins in disease we shall deal first with changes in albumin and globulin, leaving fibrinogen to be dealt with later. As regards such alterations, an *increase in total proteins* may occur in dehydration. Then both albumin and globulin are increased because of the hæmoconcentration, so that the albumin-globulin ratio remains unaltered. This is the only type of case in which an increase in albumin is found. Apart from these cases, an increase in total proteins arises only when there is an increase in one

or more of the globulin components ; the albumin either remains normal, or is reduced to a smaller extent than the globulin is increased. As a result the albumin-globulin ratio falls, sometimes markedly. *Low total proteins* are always due to a low albumin level, which is accompanied either by no increase in globulin, or by an increase which is less than the fall in albumin. Again reduced albumin-globulin ratios are found. A low serum albumin may be due to a heavy loss of albumin in the urine, to decreased formation in the liver, to increased catabolism of protein, or to insufficient protein in the food. It will be seen that a fall in albumin and a rise in globulin may almost cancel each other, in which case the albumin-globulin ratio is appreciably reduced although the total protein remains within normal limits.

A reduction in the total protein is one of the causes of œdema. The plasma colloid osmotic pressure, termed the oncotic pressure, mainly due to the plasma proteins, counteracts the effect of the capillary blood pressure, which tends to force water into the tissue spaces. When the plasma proteins are reduced sufficiently, the fall in oncotic pressure is such that water is able to pass from the plasma into the tissue spaces and so give rise to œdema. Whilst no hard and fast figures can be given, owing to the operation of other factors, œdema formation is probable when total protein falls below about 5 per cent. and albumin below about 2·5 per cent. This type of œdema is often known as nephrotic œdema since it is found in nephrosis, in which condition the heavy loss of albumin in the urine leads to low serum albumin levels, which, whilst usually in the region of 2 to 2·5 per cent., may be as low as 1 per cent. and under.

Plasma protein may also be appreciably reduced with low albumin levels in conditions in which there is severe hæmorrhage, both acute and chronic, particularly the former. The plasma volume is restored more quickly than the plasma protein, which may take some time to be brought back to normal. A similar effect is observed in shock, whether post-operative, following extensive burns, or traumatic as in crush injuries. In all these conditions there is a negative nitrogen balance due to greatly increased protein breakdown, and increase in blood urea may be found. The patient loses considerable amounts of nitrogen in the urine, about 20 grams commonly and up to and over 30 grams occasionally. A similar excessive breakdown of protein occurs in the febrile stage of acute infectious diseases. There is also increased catabolism of protein in untreated diabetes mellitus and in hyperthyroidism.

A low serum albumin is found in severe liver disease, due to the impaired ability of the liver to form albumin. The low albumin may be a factor in causing œdema in liver disease, but it should be noted that in ascites large amounts of protein may pass into the ascitic fluid. If this is tapped at intervals there is a loss of protein which also helps to produce a low plasma protein. In severe liver disease, although albumin may be found to be low with a low total protein, it is more common for the globulin to be increased so that total protein is often normal or increased.

An insufficient amount of protein in the diet may also lead to a low plasma albumin, with a low total protein also. This is the case in starvation, with hunger œdema a possible consequence. In other cases, although adequate protein is being taken in the food, absorption from the alimentary tract may be defective. This may occur in malignant disease of stomach, intestine, and pancreas, in peptic ulcer, in enteritis, in sprue and other steatorrhœas, and in vitamin deficiencies particularly of the B vitamins.

As regards increases in total protein, the increase in dehydration referred to above may be seen in all conditions in which this arises, particularly if it develops rapidly. But other factors may be present which counteract the effect of the dehydration. Thus vomiting and diarrhœa may cause impaired absorption from the intestine.

Increase in globulin occurs most commonly in advanced liver disease, in multiple myeloma, and in a number of chronic infections. In severe liver disease the globulin is often between 3 and 6 grams per 100 ml., the increase being mostly in the $\gamma$-globulin. Since the albumin is usually in the lower part of the normal range or reduced, the albumin-globulin ratio is often well below one.

Considerable interest has centred in the plasma proteins in multiple myeloma. An increase in total protein is found in just over half the cases, due entirely to an increase in the globulin fraction. Most often these have a total protein of 8 to 12 grams per cent. with a few even higher. Gutman and his co-workers (1941) have studied this condition and find considerable differences in the type of globulin responsible for the increase. In one group the increase was mainly in $\gamma$-globulin, in a second group more various patterns were seen with Bence-Jones protein present in some cases and $\beta$-globulins in others. Albumin was often low in these two groups. In a third group, essentially normal patterns of protein components were found.

An increased globulin may be observed in a considerable number of chronic infections. These include rheumatoid arthritis, tuberculosis, subacute bacterial endocarditis, kala-azar, lymphopathea venerum, Boeck's sarcoid, periarteritis nodosa, and lupus erythematosis disseminatus. In most cases the increase in globulin is small to moderate in amount, though quite considerable ones have been recorded in kala-azar and lymphopathia venerum.

For a full discussion of the variations in plasma proteins in disease, the reader is referred to Gutman (1948), Marrack and Hoch (1949) and to Muntwyler (1945) where extensive references to this subject will be found.

**Fibrinogen.** Estimations of plasma fibrinogen have not proved of great value. Like albumin, fibrinogen appears to be formed in the liver but it is only in severe liver diseases that a low plasma fibrinogen is sometimes seen, for example in acute yellow atrophy, phosphorus poisoning, and severe arsphenamine jaundice. Congenital deficiency of fibrinogen has been described so that the blood does not clot because of the absence of fibrinogen. In contrast to most other acute infections a reduced fibrinogen is found in typhoid fever.

Increases are found in most acute infections, particularly in

pneumonia and rheumatic fever, and in tubcreulosis. Fibrinogen is the component of plasma which has the greatest effect in increasing the red cell sedimentation rate which is thus much increased in conditions with raised plasma fibrinogen levels. Hence the extensive use of the sedimentation rate in rheumatic disease and in tubereulosis in assessing the severity and course of the condition. Increases in fibrinogen have been observed in nephrosis and there is a tendeney for there to be a slight increase in pregnaney.

**Cryoglobulin.** A few instances have been reported of the presenee in the plasma of a globulin which solidifies when the plasma is allowed to cool to room temperature and liquefies again on warming to 37° C. This proeess, which oeeurs slowly, can be repeated indefinitely. The term cryoglobulin has been given to this protein. It has been found in a case of multiple myeloma by Blades (1951), who gives referenees to other cases. See also the paper by MaeFarlane *et al.* (1952).

One consequenee of the presence of cryoglobulin is that it is difficult to separate plasma or serum at room temperature. This can easily be done at 37° C.

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*CHAPTER XII*

# LIPIDS

The term fats or lipids includes several groups of substances :

1. Simple fats, the esters of the fatty acids with glycerol. The term oil is given to those fats which are liquid at ordinary temperatures, for example olive oil, cod-liver oil. These should be distinguished from mineral oils such as liquid paraffin, which are hydrocarbons.

2. The phospholipids or phosphatides. These include lecithins, cephalins, and sphingomyelins, which give on hydrolysis, fatty acids, phosphoric acid, an alcohol (most commonly glycerol) and a base containing nitrogen, such as choline or ethanolamine.

3. The cerebrosides, which do not contain phosphorus and give on hydrolysis, fatty acids, an alcohol, and a carbohydrate.

4. Sterols, such as cholesterol, which do not resemble fats chemically, but are soluble in similar solvents.

## CHOLESTEROL

Cholesterol present in the blood occurs both free and as esters. Simple methods for estimating total cholesterol are available but the determination of free and ester cholesterol is much more time consuming. However, since it is sometimes important to know the ratio of free to ester cholesterol, methods for total and for free and ester cholesterol will be given.

### Determination of Total Cholesterol

Almost all methods use the Liebermann-Burchardt reaction, the green colour resulting from the reaction of cholesterol with acetic anhydride and sulphuric acid. Free cholesterol and ester cholesterol may react a little differently, so that for really accurate determinations the ester should be hydrolyzed before estimating the total cholesterol, but this is not done in the simplest methods, which give results sufficiently reliable for clinical purposes.

### Sackett's Method for Total Cholesterol (Sackett, 1925)

This is a modification of Bloor's method in which blood is added to an alcohol-ether mixture, which precipitates proteins and extracts the cholesterol. The supernatant fluid obtained on centrifuging is evaporated, the cholesterol taken up in chloroform, and determined colorimetrically by the Liebermann-Burchardt reaction.

**Reagents.** 1. Alcohol-ether mixture. Mix 3 volumes of alcohol and 1 volume of ether.

2. Acetic anhydride-sulphuric acid mixture. Mix 20 ml. of acetic

anhydride with 1 ml. of concentrated sulphuric acid. Make freshly just before use.

3. Chloroform.

4. Stock standard solution of cholesterol. Dissolve 200 mg. of pure cholesterol in chloroform and make up to 100 ml. with chloroform. Keep well stoppered.

5. Standard solution for use. Dilute 1 ml. of the stock standard to 25 ml. with chloroform. Five ml. of this solution contain 0·4 mg. of cholesterol.

**Technique.** Measure 10 to 12 ml. of the alcohol-ether mixture into a suitable centrifuge tube and add 0·2 ml. of serum, plasma, or whole blood. Cork the tube tightly and shake vigorously for about a minute. Then allow the tube to lie almost horizontally so that the precipitate is fairly evenly distributed along the tube. After standing thus for at least half an hour, centrifuge for a few minutes to get a firm deposit and decant the supernatant fluid completely into a suitable test tube. Evaporate to dryness in a water bath. Dissolve the residue after evaporation, in 5 ml. of chloroform. Into another tube measure 5 ml. of the standard cholesterol solution containing 0·4 mg. of cholesterol. To each tube add 2 ml. of the mixture of acetic anhydride and sulphuric acid. Mix and stand in the dark at 25° C. for fifteen minutes. Read at once in the colorimeter. With the photoelectric colorimeter, use a red filter, or transmission at 680 millimicrons.

**Calculation.** For the photoelectric colorimeter the result is given by

$$\text{Mg. cholesterol per 100 ml. blood} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}4 \times \frac{100}{0{\cdot}2}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 200.$$

A standard should be put up when using the photoelectric colorimeter. A standard curve can be prepared as follows, using a cholesterol solution prepared by diluting 10 ml. of the stock standard to 100 ml. with chloroform (1 ml. = 0·2 mg. cholesterol).

| | | | | | | |
|---|---|---|---|---|---|---|
| Mg. cholesterol per 100 ml. blood | 0 | 100 | 200 | 300 | 400 | 500 |
| Ml. standard solution, 1 ml. = 0·2 mg. cholesterol | 0 | 1·0 | 2·0 | 3·0 | 4·0 | 5·0 |
| Ml. chloroform | 5·0 | 4·0 | 3·0 | 2·0 | 1·0 | 0 |

Develop the colour as described for the test.

The same standard suggested for use with the photoelectric colorimeter is suitable for the visual colorimeter.

**Notes.** 1. For values over 500 mg. per 100 ml. it is advisable to use a smaller amount of blood, for example, 0·1 ml. for values between 500 and 1,000 mg. per 100 ml.

2. Serum or plasma is to be preferred to whole blood, since variations in disease are more marked in plasma than in whole blood.

However, if necessary, the determination can be carried out on 0·2 ml. of whole blood taken from a thumb or finger.

3. It is most important that the acetic anhydride-sulphuric acid mixture should be made immediately before use and that the time for the colour development should be strictly adhered to.

**Method of Myers and Wardell for Total Cholesterol** (Myers and Wardell, 1918)

In this method the blood is dried on plaster of Paris, which is then powdered and extracted with chloroform in a Soxhlet apparatus (Fig. 39). Subsequently the estimation is as described above.

**Reagents.** As above, except that plaster of Paris is required instead of the alcohol-ether mixture.

**Technique.** Place about 3 to 4 grams of plaster of Paris in a small evaporating dish, spread it out, and add 1 ml. of serum, plasma, or whole blood, drop by drop, over the surface so that it is distributed evenly in small amounts. Mix with a glass rod. Place in a drying oven at about 70° C. for at least an hour, breaking up occasionally with the glass rod. When dry, transfer the powder completely to the thimble of a Soxhlet apparatus. Place a piece of crumpled filter paper on the top of the plaster of Paris. Both thimble and paper should be fat-free. Place the thimble in the extraction part of the Soxhlet. The size of thimble used should be such that the top is higher than the siphon tube of the Soxhlet. Extract with chloroform for one to two hours. By stopping the extraction when there is a considerable volume of chloroform in the extraction chamber, it is possible to leave about 20 ml. of chloroform extract in the flask below. If not, evaporate down to this amount. Then transfer, with washing, to a 25 ml. stoppered measuring cylinder and make to the mark with chloroform. Pipette off 5 ml. of the solution and proceed with the colour development as described above, using 5 ml. of the weak standard solution as the standard.

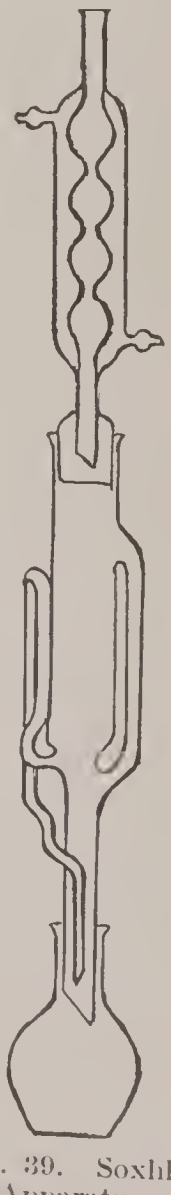

Fig. 39. Soxhlet Apparatus.

**Calculation.** This is the same as in the previous method, and the same standard curve for the photoelectric colorimeter can be used.

**Note.** It has been held that the plaster of Paris adsorbs pigments and substances other than cholesterol, which may produce some colour, so that this method is said to give lower results than that of Sackett. With serum or plasma, however, the methods appear to be in closer agreement.

**Determination of Free and Ester Cholesterol**

(Schoenheimer and Sperry, 1934 ; Sperry, 1938 ; Sobel and Mayer, 1945 ; Clarke and Marney, 1945 ; Sperry and Webb 1950).

Proteins are precipitated from serum or plasma by means of an alcohol-acetone mixture which also extracts the cholesterol and cholesterol esters. Cholesterol is then determined by means of the Liebermann-Burchardt reaction after being precipitated with digitonin before and after saponification, thus giving free and total cholesterol respectively.

**Reagents.** 1. Acetone-absolute alcohol mixture. Mix equal volumes.

2. Acetone-ether mixture. Mix 1 volume of redistilled acetone with 2 volumes of peroxide-free ether.

3. Ethyl ether. Analar.

4. Glacial acetic acid.

5. Digitonin. 0·5 per cent. solution in 50 per cent. alcohol. Dissolve 500 mg. of digitonin in 100 ml. of the alcohol at 60° C.

6. Potassium hydroxide, 10 N solution.

7. Phenolphthalein, 1 per cent. in 95 per cent. alcohol.

8. Acetic acid, 10 per cent. (v/v) solution. Dilute 10 ml. of glacial acetic acid to 100 ml. with water.

9. Concentrated sulphuric acid.

10. Acetic anhydride.

11. Stock standard solution of cholesterol. Dissolve 100 mg. of cholesterol in glacial acetic acid and make up to 100 ml. with acid.

12. Standard cholesterol solution for use. Dilute 10 ml. of the stock standard to 100 ml. with glacial acetic acid. This contains 0·1 mg. of cholesterol per ml.

**Technique.** Add 1 ml. of serum or plasma, drop by drop, with constant shaking, to about 10 ml. of the acetone-alcohol mixture in a 25 ml. stoppered measuring cylinder. Heat carefully in boiling water, with constant shaking, until the mixture just comes to the boil. On reaching boiling point, remove from the boiling water, but continue shaking for about a minute. Add a further 10 ml. of the mixture and repeat the heating. Then cool and make up to the mark with further acetone-alcohol mixture. Mix well and filter into a dry test tube. Keep the funnel covered and work rapidly to avoid loss by evaporation.

Clark and Marney allow extraction to take place overnight at room temperature. They add 2 ml. of serum or plasma drop by drop to about 40 ml. of acetone-alcohol mixture in a 50 ml. stoppered cylinder. Add this mixture to just below the mark, stand overnight and make up to the mark next morning.

The *free cholesterol digitonide is precipitated* as follows. Pipette 5 ml. of the above filtrate into a tapered centrifuge tube of about 15 ml. volume. Add 2 drops of the 10 per cent. acetic acid and 2·5 ml. of the digitonin solution and stir well with a thin glass rod. Leave the rod in the tube and stand in a closed jar overnight. Remove from the jar next morning, take the stirring rod out of the tube and stand it in a rack so that it can be used later in the same estima-

tion and any precipitate attached to it will not be lost. Centrifuge the tube at about 3,000 r.p.m. for about ten to fifteen minutes. Remove the supernatant fluid, add 4 ml. of alcohol-acetone mixture to the tube and mix the precipitate well with this by means of the stirring rod already used. Remove the rod as before and centrifuge again. Remove the supernatant fluid and wash the precipitate in the same way twice with 4 ml. of ether. After the last washing, dry the precipitate by placing for a few minutes in a water bath at 40° to 50° C. Replace the rod in the tube. The tube is ready for colour development.

The *total cholesterol digitonide is prepared* similarly after saponifying as follows. Pipette 2·5 ml. of the filtrate from the alcohol-acetone extraction into a similar centrifuge tube and add 2 drops of 10 N potassium hydroxide. Stir well with a thin glass rod until the alkali is thoroughly mixed with the alcohol-acetone extract and place in a closed jar in an incubator at 37° C. for half an hour. A layer of sand can be placed in the jar and heated to about 45° C. before standing the tube in it and placing the whole in the incubator. Then remove the tube from the jar, cool, and add alcohol-acetone mixture to 5 ml. Add a drop of phenolphthalein and make slightly acid with 10 per cent. acetic acid, adding 1 or 2 drops more acid after the red colour of the phenolphthalein has disappeared. Add 2·5 ml. of the digitonin solution, stir well, stand overnight as above and proceed with the separation and washing of the precipitate in the same way as there described. Only one washing with ether is necessary.

The colour can now be developed from the two precipitates. Add 2 ml. of glacial acetic acid to each tube and dissolve the precipitate with the aid of heat. Place the tubes in boiling water and mix continuously until the precipitate dissolves.

When dissolved, transfer the tubes to a water bath at 25° C. out of contact with light, and, whilst temperature adjustment is taking place, make up some fresh acetic anhydride-sulphuric acid mixture in the ratio of 20 ml. of anhydride to 1 ml. of acid. This should not be used if more than thirty minutes old. When the tubes are at 25° C. add to each, 4 ml. of the mixture, mix with the glass rod and stand in the 25° C. bath in the dark for exactly thirty minutes. Set up a blank with 2 ml. of glacial acetic acid and 4 ml. of the anhydride-acid mixture at the same time. Also put up a suitable standard. Two ml. of the weak standard with 4 ml. of anhydride-acid mixture is satisfactory. Compare in the colorimeter or read in the photoelectric colorimeter using an orange filter, transmission at 620 millimicrons. Make the reading as quickly as possible because of the effect of light on the colour.

**Calculation.** With the photoelectric colorimeter, since there are 0·2 mg. cholesterol in the standard

Mg. total cholesterol per 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}2 \times \frac{100}{0{\cdot}1}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 200.$$

$$\text{Mg. free cholesterol per 100 ml. serum} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 100.$$

To prepare a standard curve for the photoelectric colorimeter prepare a solution containing 0·5 mg. cholesterol per ml. by diluting the stock standard 1 in 2 with glacial acetic acid and set up the following tubes :

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Mg. total cholesterol per 100 ml. serum | 0 | 100 | 200 | 300 | 400 | 500 | 600 |
| Mg. free cholesterol per 100 ml. serum | 0 | 50 | 100 | 150 | 200 | 250 | 300 |
| Ml. standard containing 0·5 mg. cholesterol per ml. | 0 | 0·2 | 0·4 | 0·6 | 0·8 | 1·0 | 1·2 |
| Ml. glacial acetic acid | 2·0 | 1·8 | 1·6 | 1·4 | 1·2 | 1·0 | 0·8 |

Add 4 ml. of acetic anhydride-sulphuric acid mixture as above.

For high values of plasma cholesterol use smaller amounts of the alcohol-acetone extract, making up to the volumes given above with further alcohol-acetone mixture. The volume of acetic acid used to dissolve the precipitates may be varied provided twice its volume of anhydride-acid mixture is added.

INTERPRETATION

**Total Cholesterol.** It is generally agreed that the normal total cholesterol content of blood lies between rather wide limits, though different values for these limits have been given by different workers. Perhaps the best figures for this normal range are from 150 to 240 mg. per 100 ml., though occasionally figures are obtained below and above this range. The range of variation in the individual is narrower. The blood cholesterol does not appear to be much altered after meals. It increases slowly with age. In pregnancy there is an increase which may reach 20 to 25 per cent. of the normal value.

*Increases* in cholesterol occur in a number of conditions. This increase is more marked in plasma than in whole blood. Hypercholesterolæmia is found most characteristically in nephrosis, diabetes mellitus, obstructive jaundice, myxœdema, and in xanthomatosis, and less consistently in a number of other conditions.

In *nephrosis*, the earlier stages of Type II nephritis of Ellis, when œdema is present, values of up to 600 to 700 mg. per 100 ml. are common and may occasionally reach and exceed 1,000 mg. per cent. Otherwise, in chronic glomerulonephritis the cholesterol is within normal limits or but slightly increased. This is also the case in most patients with acute glomerulonephritis at least in the early phase, but should the disease pass into the subacute stage, marked increases occur.

Figures up to 400 and 500 mg. per cent. are commonly found in *diabetes mellitus* when treatment is inadequate. In *jaundice*, increase occurs most commonly in obstructive jaundice due to obstruction of the large ducts. The increase is said to parallel the increase in

serum bilirubin. In parenchymatous liver disease findings are more variable. Whilst serum cholesterol is normal in many cases, increases are found in an appreciable proportion, particularly in the recovery stages of the condition. Very high figures are found in xanthomatous biliary cirrhosis.

An increase is commonly found in *myxœdema* the rise roughly paralleling the fall in basal metabolic rate. Values up to 500 to 700 mg. per 100 ml. are seen and the estimation may give valuable help in diagnosis. Small increases may be found in hypopituitarism, usually in the range 250 to 350. Administration of thyroxin leads to a marked fall in the serum cholesterol.

*Xanthomatosis* is frequently associated with an increase in blood cholesterol. The subject has been discussed at length by Thannhauser (1947, 1950). He distinguishes between primary and secondary xanthomatosis. The former is further subdivided into two groups, in one of which there is raised serum cholesterol, whereas in the other it is within normal limits, the deposits being composed of other lipids. In secondary xanthomatosis the condition is found to accompany a hypercholesterolæmia arising from one of the conditions mentioned above, such as diabetes mellitus and nephrosis.

Moderate increases in cholesterol are a rather frequent finding in coronary thrombosis and in angina pectoris.

*Decreases* in blood cholesterol are not so well defined. Because of the rather wide normal range an appreciable fall may occur in a particular individual before the blood level goes below the lower limits of normal. Thus the effect of *hyperthyroidism* is to reduce the serum cholesterol and values as low as 80 to 100 mg. per 100 ml. may be seen. However, quite a number are found to have a serum cholesterol within the normal range. The estimation in this condition must be regarded as being of very limited value as a general rule. It has been used to follow the effect of thiouracil therapy, which brings about an increase in cholesterol.

Low values are not infrequently obtained in pernicious anæmia and in other anæmias, and sometimes also in hæmolytic jaundice, falling below 100 mg. per 100 ml. in some cases. Similar findings may be seen in acute infections, in severe wasting and in a number of terminal states.

**Ratio of Free to Ester Cholesterol.** Free cholesterol normally forms about 30 per cent. of the total cholesterol, ranging from 20 to 40 per cent., so that the ester fraction forms 70 per cent. of the total, ranging from 60 to 80 per cent. Whilst changes in total cholesterol do not involve any change in the percentage of free and ester cholesterol in diabetes, nephrosis, and myxœdema, in liver disease the percentage of ester cholesterol decreases. The tendency when liver damage is present, as in infective hepatitis during the earlier phases whilst the jaundice is developing, is that the total cholesterol either remains normal or falls a little while the proportion of ester cholesterol falls appreciably and may reach very low levels. As the condition improves, the total cholesterol rises as does the proportion of ester cholesterol, so that the values for the total cholesterol are high for

some time after the jaundice has disappeared. In severe acute liver necrosis as in acute yellow atrophy, the total serum cholesterol is usually low and may fall below 100 mg. per 100 ml., whilst there is a marked reduction in the percentage present as esters. In obstructive jaundice due to obstruction of the large ducts there is marked increase in the total cholesterol. It has been stated that the ratio of free to ester cholesterol is comparatively little changed. However, there is evidence that an increase in this ratio occurs in a considerable proportion of these cases (see Man *et al.* 1945). In xanthomatous biliary cirrhosis the increase is almost entirely in the free cholesterol.

## PHOSPHOLIPIDS

### Determination of Phospholipids, Lipid Phosphorus

The lipids are extracted into an alcohol-ether mixture and digested with sulphuric acid and hydrogen peroxide as introduced by Youngberg and Youngberg (1930). The phosphorus, now present as phosphate is then determined colorimetrically by the method for inorganic phosphorus.

**Reagents.** 1. Alcohol-ether mixture. Mix 3 volumes of alcohol and 1 volume of ether.

2. Sulphuric acid, 10 N solution.

3. Hydrogen peroxide, 100 volumes (30 per cent.).

Other reagents are almost the same as for the determination of inorganic phosphorus (see p. 302).

**Technique.** Measure about 16 ml. of the alcohol-ether mixture into a 6 × 1 inch test tube and add drop by drop, with constant shaking, 1 ml. of serum or plasma. Heat carefully to boiling in a boiling water bath. Cool, make up to 20 ml. with further alcohol-ether mixture. Mix and filter, taking care to avoid loss by evaporation. If total fatty acids are also being determined (p. 192) use some of the same alcohol-ether extract.

Pipette 8 ml. of the filtrate (equivalent to 0·4 ml. of serum) into another 6 × 1 inch tube and evaporate to dryness. Add 1 ml. of 10 N sulphuric acid and heat gently. When the mixture turns brown remove the flame and add a drop of hydrogen peroxide. Heat further and repeat the addition of hydrogen peroxide at intervals until the digestion is complete and the liquid colourless. Add a few ml. of water and boil for a few seconds.

Estimate the phosphorus by the method of Fiske and Subarrow described on p. 302. Transfer with washing to a 10 ml. stoppered cylinder. Add 1 ml. of 2·5 per cent. ammonium molybdate solution, and 0·4 ml. of amino-naphtholsulphonic acid and make up to the mark. Carry through a complete reagent blank. A standard can be prepared using 5 ml. of a solution containing 0·04 mg. of phosphorus. Develop the colour from this in the same way as for the unknown. Read in the photoelectric colorimeter against a standard blank, using a red filter or transmission at 680 millimicrons. Alternatively, put through a fully digested standard in the same way as the test and read both against the reagent blank.

**Calculation.** Since 8 ml. of the filtrate is equivalent to 0·4 ml. of the serum and the standard contains 0·04 mg. of phosphorus, for the photoelectric colorimeter.

Mg. phospholipid phosphorus

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{100}{0{\cdot}4} \times 0{\cdot}04$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 10.$$

A standard curve can be prepared as follows, using a standard prepared by dissolving 0·351 gram of potassium dihydrogen phosphate in water, adding 10 ml. of 10 N sulphuric acid and making up to a litre with water. One ml. contains 0·08 mg. of phosphorus.

| Mg. phospholipid P. per 100 ml. serum | 0 | 5 | 10 | 15 | 20 |
|---|---|---|---|---|---|
| Ml. phosphate standard . . . | 0 | 0·25 | 0·5 | 0·75 | 1·0 |

Develop the colour as described for the test.

INTERPRETATION

Normal values for plasma lipid phosphorus in the post-absorptive state are given as 6 to 11 mg. per 100 ml., averaging 9 mg. per 100 ml. There is rather more in whole blood since there is about twice as much in the cells as in plasma. To convert into phospholipids multiply by twenty-five. About half the phospholipid of plasma is lecithin. Thannhauser, Benotti and Reinstein (1939), and Erickson *et al.*, (1940) have published methods for determining lecithin, cephalin, and sphingomyelin in blood and tissues. The plasma phospholipid bears quite a close relationship to the plasma cholesterol. The average ratio of cholesterol to lipid phosphorus when cholesterol is normal is 21 (Peters and Man, 1943 (*b*) ). Phospholipids increase in those conditions in which there is an increase in cholesterol, though the increase is usually smaller. This applies to liver and biliary tract diseases, diabetes mellitus, and myxœdema.

## TOTAL FATTY ACIDS IN BLOOD

**Determination of Total Fatty Acids in Serum or Whole Blood** (Stoddart and Drury, 1929 ; Peters and Van Slyke, 1932)

The fats are extracted with an alcohol-ether mixture which precipitates the proteins. They are then saponified, the fatty acids liberated by acidification, washed, and determined by titration with sodium hydroxide.

**Reagents.** 1. Alcohol-ether mixture. Prepare by mixing 3 parts of 95 per cent. ethyl alcohol (aldehyde free) and 1 part of ether (peroxide free).

Aldehyde-free alcohol can be prepared as follows. Reflux about 750 to 1,000 ml. of 95 per cent. alcohol with 35 to 50 grams of potassium hydroxide for about six hours. Distil the alcohol into a side-

arm flask the outlet of which is protected by a calcium chloride tube to prevent addition of water to the alcohol.

To obtain peroxide-free ether, redistil the ether through an eight-bulb piece condenser column. To test for peroxide, shake some ether with one half its volume of 10 per cent. potassium iodide slightly acidified with sulphuric acid. If more than a faint yellow colour develops reject the ether or repurify.

2. Saturated solution of sodium hydroxide (carbonate-free). Mix equal weights of water and pure hydroxide, cover, and stand for about two days for the carbonate to settle out. Decant the solution.

3. Hydrochloric acid, 30 per cent. (v/v).

4. Sodium hydroxide, 10 per cent. solution.

5. One per cent. phenolphthalein in 95 per cent. alcohol.

6. Thymol blue, 0·3 per cent. solution in 50 per cent. alcohol.

7. Sodium hydroxide, 0·02 N. Prepare from a saturated solution of carbonate-free sodium hydroxide.

8. Sodium chloride, 5 per cent. solution neutral to methyl red.

9. Methyl red. A saturated solution in 50 per cent. alcohol.

**Technique.** Pipette 5 ml. of the specimen, which may be either whole blood, plasma or serum into 75 ml. of the alcohol-ether mixture in a 100 ml. volumetric flask. Add slowly, drop by drop, keeping the liquid rotating so that there will be no formation of large aggregates of precipitate. Place the flask in boiling water whilst shaking continuously to avoid superheating, until the liquid begins to boil. Cool to room temperature, make up to the 100 ml. mark with further alcohol-ether mixture and mix well. Filter through a fat-free filter paper.

Measure 75 ml. of the filtrate and evaporate in a 100 ml. beaker adding the liquid gradually. To prevent bumping add a few grams of coarse sand previously boiled with acid, then washed, dried, and finally extracted with ether. Place on an electric plate at a low heat or on a water bath so that the liquid does not boil perceptibly and allow the ether to evaporate off. Then evaporate down to about 30 ml. Add 0·1 ml. of saturated carbonate-free sodium hydroxide. Mix, add a few grains more sand, cover with a watch glass and boil gently for twenty to thirty minutes. This saponifies the fats.

Remove the watch glass, drop in a small piece of litmus and make acid with 30 per cent. hydrochloric acid. Then make just alkaline with 10 per cent. sodium hydroxide. This avoids an excess of alkali on evaporating to dryness which is then done to remove the alcohol. Add 15 ml. of water, heat on the water bath with stirring to dissolve the soaps. Whilst still hot, add a drop of thymol blue and make acid with 30 per cent. hydrochloric acid, shown by a faint pink colour. After standing for ten minutes in cold water swirl almost continuously for five minutes so as to get a better separation of the fatty acids.

Then filter the fatty acids through a 10 ml. Gooch crucible containing a paper pulp mat. These filters are made as follows. Fix up the crucible in a 500 ml. suction flask. Prepare a paper pulp emulsion by shaking a piece of soft filter paper vigorously in about 300 ml. of distilled water. Pour immediately into the crucible with

strong suction on. Repeat shaking and filtering until a layer about 1 mm. thick is obtained. With a glass rod tamp down carefully all over. Allow the larger masses of filter pulp fibres in the emulsion to settle out and pour on successive amounts of the thin upper suspension of isolated shreds, keeping a strong suction on and tamping down occasionally until the filter is dense enough to offer a definite resistance to the suction. Remove the crucible from its rubber holder and dry in an air oven at 110° C. for fifteen minutes. Cool before using.

For the filtration place the crucible in its washer in a funnel and place a test tube under the funnel. Pour some of the fatty acid suspension into the crucible. If it does not filter clear, put it through the crucible again. If filtration does not begin within a few minutes change the crucible to the funnel in a filter flask and apply suction gently. When filtration has begun continue without suction. When the fatty acid suspension is filtered and has drained, wash with 4 ml. portions of 5 per cent. sodium chloride solution. Wash down the sides of the beaker thoroughly and pour the rinsings into the Gooch crucible. Continue washing until the filtrate from one washing requires not more than 0·05 ml. of the 0·02 N sodium hydroxide to neutralize it to phenolphthalein. Three washings usually suffice.

Replace the crucible in the suction flask and dissolve the fatty acids in hot 95 per cent. alcohol. To do so, run into the beaker used for precipitating the fatty acids, 5 ml. of the alcohol washing the walls whilst doing so. Bring to the boil and pour into the crucible. Repeat the washing with two further 3 ml. portions of hot alcohol.

After adding a little sand, boil for a minute, cool, note the volume and after adding 3 drops of thymol blue as indicator, titrate with 0·02 N sodium hydroxide to a pure blue colour. Titrate as a blank, 10 ml. of alcohol evaporated down to the same volume as that of the boiled solution of fatty acids.

**Calculation.** Let T = titration of the test in ml. of 0·02 N sodium hydroxide, and B = the titration of the blank. It is necessary to subtract from T a further correction of 0·005 ml. for each ml. of 0·02 N sodium hydroxide used, to correct for the effect on the end point of the water added in the titration.

So ml. of 0·02 N sodium hydroxide required to titrate the fatty acids = 0·995 T − B.

Use as a factor for fatty acids 277 which is the average molecular weight of the fatty acids present in blood.

Since the alkali is 0·02 N and 5 ml. of blood were taken and 75 ml. of filtrate used

Mg. fatty acids per 100 ml. of blood

$$= (0{\cdot}995\ \mathrm{T} - \mathrm{B}) \times \frac{277}{50} \times \frac{100}{5} \times \frac{100}{75}$$
$$= (0{\cdot}995\ \mathrm{T} - \mathrm{B}) \times 148.$$

For ordinary purposes the correction factor 0·995 can be ignored. So

Mg. fatty acids per 100 ml. blood = 148 × (T − B)
Millimols fatty acids per 100 ml. blood = 0·533 × (T − B).

**Note.** The method of filtration described is that used by Stoddart and Drury. However, filtration through fat-free filter paper (for example, Whatman No. 43) can be used and satisfactory results obtained.

*Other Method.* Bloor (1922, 1928) extracts the lipids into an alcohol-ether mixture. They are then oxidized by means of potassium dichromate, the excess of which is measured iodometrically.

INTERPRETATION

The question of the blood lipids was reviewed by Thannhauser (1947). Total fatty acids of plasma or serum, when fasting, range from 200 to 450 mg. per 100 ml. (see Erickson, 1937). A higher figure for the upper limits has been given by some workers (see Peters and Man, 1943 (*a*) ). These fatty acids are derived from neutral fats, of which there are 0 to 150 mg. per 100 ml., cholesterol esters, phospholipids, and cerebrosides. Total fatty acids as thus estimated, are more variable than cholesterol and phospholipids. The determination cannot be used to give an indication of variations in neutral fat, since the total fatty acids may be much increased because of increase in phospholipids and cholesterol esters in cases in which neutral fat is very low. No reliable method is available for determining neutral fat when cholesterol esters and phospholipids are also present. A figure for the neutral fat is usually obtained by subtracting the fatty acids present as cholesterol esters and phospholipids from the total fatty acids as determined above. Thannhauser and Reinstein (1942) suggest the formula

Mg. neutral fat per 100 ml. = [Total fatty acids in mg. per 100 ml. − (0·72 × cholesterol esters in mg. per 100 ml. + 0·69 × phospholipids in mg. per 100 ml.)] × 1·04.

The small amount of cerebrosides present is thus included in the neutral fat, of which, however, it forms only a few per cent.

All determinations should be done fasting, since there is a marked increase after meals. The peak of this occurs some three to five hours after the meal, at which time the total fatty acids may be more than twice as great as in the fasting state.

Strictly speaking the term hyperlipæmia should be given to those sera or plasmas in which there is an increase in neutral fat. It is these which appear milky in appearance, in many cases sufficiently for this to be noticeable even on inspection of whole blood. Increase of other fatty acid-containing compounds, phospholipids and cholesterol esters, or of cholesterol, does not produce a milky plasma. However, the term hyperlipæmia is sometimes used in a wider sense to refer to an increase in any of the lipid constituents.

Neutral fat may be increased in diabetes mellitus, and less markedly in nephrosis. An increase has also been reported in myxœdema by several workers, but Peters and Man (1943 (*b*) ) have found it to be almost uninfluenced by the thyroid. An essential or idiopathic hyperlipæmia has been described, in which neutral fat and other lipids

are increased, the increase not being referable to any known cause. Such cases show a quick response to low fat diets.

Hyperlipæmia has been regarded as due to a slow rate of removal from the blood, retention hyperlipæmia, or to an increased rate of mobilization from the fat depots, transportation hyperlipæmia. A good example of the second is untreated diabetes mellitus, which responds to insulin but not to a low fat diet. The essential hyperlipæmia, which may be accompanied by a slight glycosuria, is apparently of the former type in responding to a low fat diet but not to insulin.

### Total Lipids

This term is given to the sum of the blood lipids, that is the sum of the free and ester cholesterol, phospholipids and neutral fat. Peters and Van Slyke (1946) give the range in normal persons as 360 to 820 mg. per 100 ml. with a mean of 570 mg. per 100 ml. The figure for the total lipids is of relatively little value in itself. To assess the significance of variations we need to know what changes there are in the constituents of which it is composed.

### Lipoidoses

This term has been given to the group of diseases in which one or more of the various lipids are deposited in certain organs or tissues, usually in reticulo-endothelial cells. We have already referred to cases in which cholesterol forms the major part of such deposits, and to which the name xanthomatosis is given. These may be found in all conditions in which the plasma cholesterol is increased. Other lipids as well as cholesterol may be found in such deposits, often without any changes in the plasma lipids. For fuller discussion of these conditions, see Thannhauser, 1950, and also Peters and Van Slyke, 1946 ; Sobotka, 1942 ; and Sperry, 1942.

In *Hand-Schüller-Christian's* disease the chief lipid deposited is cholesterol, both free and ester, though serum cholesterol is not increased ; in *Gaucher's disease* there are accumulations of cerebrosides (of kerasin) ; in *Niemann-Pick's* disease the deposits are of phospholipids, containing, according to Thannhauser, sphingomyelin but not lecithin or cephalin. In Niemann-Piek's disease, though many workers have reported plasma lipids normal, some have found a small to moderate increase.

*Tay-Sach's disease* resembles Niemann-Pick's disease in that the lipid is mainly deposited in the brain. From the deposit in Tay-Sach's disease a galactoside containing sphingosine and galactose has been isolated (Klenk and Schumann, 1940). The plasma lipids are normal.

## FAT IN URINE

Fat has been said to occur in urine (*lipuria*) after large amounts of fat have been taken; in conditions such as nephrosis and diabetes mellitus in which there is an increase in blood fat : and following

injuries in which there has been considerable crushing of subcutaneous fat. Lipuria, if it occurs, is certainly a rare condition. There are many ways in which urine may become contaminated with extraneous fat, as, for example, from liquid paraffin used as a laxative, lubricants used in passing catheters, and even from the deliberate addition of fat.

A particular example of lipuria is *chyluria*, the term given to milky urines resulting from admixture with chyle from ruptured lymphatics in the kidneys or bladder. In chyluria there are also red blood cells present, and in some cases there may be enough fibrinogen to form clots. The amount of fat present may reach a few per cent. but varies considerably with changes in fat absorption. The fat is said to be more finely dispersed in chyluria, than in other cases of lipuria. For recognizing this condition Sudan III has been given with fat in the food. Thus Harrison (1947) gives 100 mg. with 10 grams of butter. The Sudan III is excreted with the fat in the urine and can be extracted into ether.

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## *CHAPTER XIII*

# TESTS OF GASTRIC FUNCTION. OCCULT BLOOD

The chief constituents of gastric juice are : (1) Hydrochloric acid, secreted by the parietal cells. (2) Pepsinogen, secreted by the chief cells. This is activated to pepsin by the acid which also provides the acid medium required by this enzyme for its action on proteins. These are partially broken down to peptones and proteoses. (3) Rennin, which clots milk by converting the milk protein caseinogen to casein. (4) The hæmopoietic factor, absence of which is responsible for the development of pernicious anæmia. (5) Mucus.

The gastric secretion is produced in response to (1) Psychic factors, sight, taste, smell, and even in extreme circumstances the thought of food. This is mediated by the vagus nerve. (2) Gastrin, a hormone secreted by the gastric mucosa when food is present in the stomach. (3) The presence of some products of digestion in the intestine.

Insulin by causing hypoglyæmia stimulates the vagus to increase the output of both acid and pepsin, whilst histamine is a powerful stimulant of acid secretion. Alcohol in moderate concentration also stimulates the production of acid. While each of these substances has been used in investigating gastric function, meals composed of ordinary foods have been more frequently used. One of the earliest of these was the *Ewald One Hour Meal.* After a period of fasting a meal of toast, usually without butter, and tea without milk, was given. An hour later a stomach tube was passed and the stomach contents removed. Tests similar to those for the fractional test meal were used. This test has fallen into almost complete disuse since more information is obtained by collecting samples at frequent intervals during the digestive period as is done in the fractional test meal of Rehfuss.

## THE FRACTIONAL TEST MEAL OF REHFUSS

The stomach tube is a thin rubber one, 4 mm. external diameter, two forms of which are in use. The Rehfuss tube has an uncovered metal end with openings about the size of the bore of the tube (Fig. 40 (*a*) ), the Ryle tube a rubber-covered end containing a small weight of lead, the holes being in the rubber tube a short distance from the end (Fig. 40 (*b*) ). Both tubes have markings to indicate how far the tube has been swallowed. These take the form of black rings. When the single ring reaches the lips, sufficient tube has been swallowed in an average case for the end to reach to the cardiac orifice ; with the double ring at the lips the end of the tube should be in the body of the stomach, sometimes almost to the pylorus. On

longer tubes there is sometimes a triple ring which marks a length of tube long enough to allow the end to pass spontaneously into the duodenum later in the meal. The choice of tube is a matter of personal preference. The Ryle tube would seem to be a little easier to swallow and a little less likely to cause trauma, but tends to block more easily than the Rehfuss tube.

The *meal* most used is one of oatmeal gruel. This is prepared by adding two tablespoonfuls of oatmeal to a quart of water, simmering down to about a pint, and straining through muslin. Add a little sugar if it is desired to make the gruel more palatable. Salt should not be added, particularly if chlorides are to be determined. The meal should not be too thin but as thick as is consistent with the patient being able to drink it.

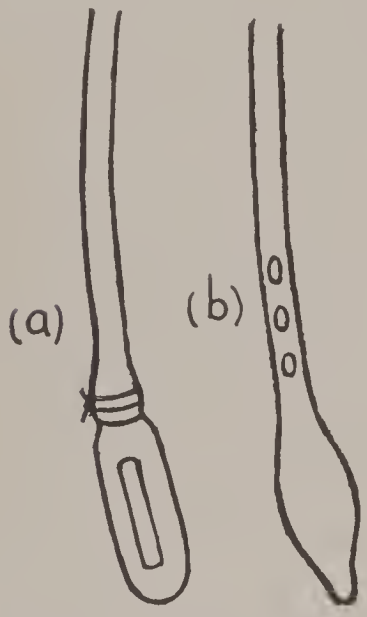

FIG. 40. Stomach Tubes.

### Method of Carrying out the Test

Instruct the patient not to eat or drink anything during the twelve hours immediately preceding the test. This means taking nothing after supper at 9 to 10 p.m. on the evening before the test is done. Sometimes a couple of charcoal biscuits are given with milk for this supper. The charcoal is easily recognized in the fasting contents next morning if still present and shows that there is abnormal retention of stomach contents.

Begin the test about breakfast time next morning. Before use place the tube for a short time in boiling water, withdraw the burner and leave the tube in the water until required. In passing the tube, which may be lubricated with liquid paraffin or glycerol, ask the patient to open the mouth and hold the tongue forward a little. Place the tip of the tube on the back of the tongue, get the patient to say " Ah," push the tube forward into the throat and ask the patient to swallow, at the same time pushing the tube forward. Once the tube has passed the pharynx, in most cases it will go down fairly easily, being pushed forward each time the patient swallows. There may be some retching in the earlier stages. This is often due to pharyngeal stimulation. Do not hurry the patient unduly. Upward pressure applied beneath the lower jaw may be used to prevent the tube from being vomited, or the patient should shut the mouth tightly and take a few deep breaths through the nose. In a very few cases the retching continues so that it is difficult to keep the tube in position, and even impossible to pass it. In some cases it is possible to pass a Ryle's tube through the nose.

In most cases the tube is passed to the double ring, a distance of about 50 cm. The best distance will vary somewhat from patient to patient, but the appearance of the aspirated contents, and the ease with which samples are obtained, will show the practised operator

whether the tube is satisfactorily placed. When the tube is in position every endeavour should be made to keep it so, and the patient instructed accordingly. Saliva should not be swallowed, but expectorated if it is possible for this to be done without disturbing the position of the tube unduly.

Remove the resting contents. A 10 ml. record type syringe is satisfactory. Take a few minutes to do this, moving the patient from side to side so as to make sure that the stomach has been emptied as completely as possible. Measure the volume, and keep for examination with the later specimens. Clip the tube and give the patient a pint of the oatmeal gruel to drink. Note the time, and remove about 10 ml. of stomach contents every quarter hour, so long as it is possible to do so, up to two and a half hours after the meal was taken. Sometimes the stomach empties before this time is reached. When this is not the case, some workers empty the stomach at the end of two and a half hours, and measure the volume of residual contents. Then gently withdraw the tube. In the very small number of cases in which the end of the tube sticks on withdrawal, instruct the patient to swallow, or give a little water to drink, and pull gently while either of these is being done.

Take particular care to obtain reliable specimens. These are best collected by a person who has had some experience of doing so and has become familiar with the appearance of the specimens. There are three common errors from which unsatisfactory samples may result.

1. The tube may become blocked with mucus or food residues so that the stomach is wrongly assumed to be empty. Careful injection of a little air, or a few ml. of water, may be sufficient to remove the obstruction so that specimens can again be obtained.

2. The tube may not be sited properly in the stomach so that either no specimens are obtained, or, if saliva is being swallowed, a series of samples almost entirely composed of saliva are taken and sent for analysis. A wrong finding of achlorhydria may result. X-ray siting has been used, and radio-opaque tubing is now available. Experienced workers are able to place the tube correctly in most cases.

3. Too much tubing may be swallowed with the result that later in the test the tube passes into the duodenum and duodenal contents, heavily bile stained, are obtained. This is unlikely to occur if the double mark is kept at the teeth. While regurgitation of bile may occur during a test meal, it is not common. When a heavily bile-stained specimen is obtained the tube should be withdrawn a little, and a further specimen taken and examined. Specimens free from bile can then often be obtained.

Oatmeal gruel has certain advantages as a test meal. It is easily aspirated through the narrow tube, and abnormal substances such as bile, blood, and excessive mucus, can be detected by simple inspection in most cases. Also, filtering gives a clear colourless filtrate which is suitable for titrating the acid present. A reasonably standard response is given by the stomach to such a meal.

### Examination of the Specimens

Filter the specimens when obtained. Filtration is sufficiently rapid in the majority of cases, but may be slow occasionally, particularly with specimens in which there is no free acid and which contain much mucus. This often occurs when much saliva is swallowed, when in any case the specimens are of doubtful value.

For specimens containing much mucus, Harrison (1947) has suggested placing the specimen in a centrifuge tube with a fairly tight plug of cotton wool on top of the liquid. On centrifuging hard, this plug is forced downwards, leaving clear fluid on top whilst the mucus remains below the plug.

*Test for starch* by adding a drop or two of iodine solution to the material on the filter paper. The amount of starch present gives a rough idea of the rate of emptying of the stomach in some cases.

*Note the Presence of any Blood or Bile.* These can usually be detected by simple inspection. Should doubt arise, apply chemical tests. See pp. 216–9 for tests for blood ; p. 232 for tests for bile. However, the benzidine test is often too sensitive. Inspection is satisfactory in most cases.

Inspect also for the presence of any excessive amounts of *mucus*. This is best done when the specimens are being taken.

Note any odour. The fermentation which gives rise to lactic acid and other organic acids may produce a rather characteristic smell with which technicians soon become familiar.

If charcoal was given the previous evening look to see if any is still present in the resting contents.

### Titration for Free and Total Acid

This is best done by titrating a portion of the filtered specimens with a standard solution of sodium hydroxide, using two indicators in succession. The indicators most commonly used are methyl orange or Töpfer's reagent, which change from red to yellow from *p*H 2·9 to 4·4, then phenolphthalein changing back to red from *p*H 8·3 to 10·0. The first titration to about *p*H 4 measures the amount of free hydrochloric acid present, the *free acid*. From this point to the colour change with phenolphthalein some protein hydrochloride and any organic acids present will be titrated and there will be some conversion of acid to alkaline phosphate. Proteins present include mucin in the gastric secretion and protein in the meal given. The difference between the two titrations is sometimes termed the *combined acid*, but this is only correct so far as it refers to protein hydrochloride which is never more than partly the case. The complete titration is said to give the *total acid*.

**Reagents.** 1. N/100 sodium hydroxide. Do not leave this exposed to the atmosphere ; prepare freshly for use from stock N/10 solution or protect with a soda lime tube.

2. Töpfer's reagent, 0·5 per cent. dimethylaminoazobenzene in absolute alcohol, or a 0·1 per cent. aqueous solution of methyl orange.

3. One per cent. solution of phenolphthalein in 50 per cent. alcohol.

**Technique.** Pipette 1 ml. of filtered gastric contents into a small beaker (one about 60 ml. volume is suitable), add 2 to 3 drops of Töpfer's reagent or methyl orange and titrate with N/100 sodium hydroxide until all trace of the red colour disappears and the colour is yellowish orange. Note the volume of alkali added. Then add 2 or 3 drops of phenolphthalein and continue titrating until a definite red tinge reappears. The most usual difference between the two colour changes is about 1 ml. Again read the burette and so obtain the total volume of alkali added. It is better not to mix the two indicators but to add them separately.

If a yellow colour is obtained on adding Töpfer's reagent or methyl orange, no free acid is present. Add the phenolphthalein and titrate the combined acid. This then equals the total acid.

Express the results as

Ml. N/10 hydrochloric acid per 100 ml. of gastric contents.

To do this it is only necessary to multiply the actual titrations in ml. by 10. Plot two curves which show the course of the free and total acid during the meal.

**Note.** Thymol blue has also been used as indicator. It has the advantage of having two colour changes, one from red to yellow at *p*H 1·2 to 2·8, the other from yellow to blue at *p*H 8·0 to 9·5. Titration to the first colour change has been used to measure the free acid, titration to the second the total acid, but since the change from red to yellow occurs at a more acid *p*H than with Töpfer's reagent or methyl orange, small amounts of free acid may not be detected. A *p*H of 2·0 corresponds to N/100 acid and so equals 10 units as measured above, whilst *p*H 3 equals only 1 unit and *p*H 4 only 0·1 unit. However, when considerable amounts of organic acids are present (see below) it is less likely that the *p*H will be low enough to give a misleading impression that free hydrochloric acid is present with thymol blue than with Töpfer's reagent or methyl orange.

**Effect of Organic Acids on the Titration.** The possible occurrence of organic acids should be remembered. Lactic acid is the one found in largest quantities but butyric and other acids may be present. When there are large amounts of such organic acids in the resting contents the *p*H may be low enough for an acid reaction to be given with the indicator used to measure the free acid even when no free hydrochloric acid is present. In such circumstances it may be necessary to determine whether free hydrochloric acid is actually present. The Günzberg test is used for this purpose. However, the presence of organic acids can generally be recognized since when much organic acid is present the "combined acid" will be found to be much greater than usual; that is, the difference between the titrations of free acid and total acid will be increased. Furthermore, the colour change of the first indicator will occur more slowly so that the exact end point is less easily ascertained. When this occurs, tests for lactic acid should be carried out. These and the Günzberg test will be described.

### Günzberg Test for Hydrochloric Acid

**Günzberg's Reagent.** Dissolve 2 grams of phloroglucinol and 1 gram of vanillin in 100 ml. of 95 per cent. alcohol. Prepare freshly. Alternatively keep two 10 per cent. solutions of phloroglucinol and vanillin in alcohol and mix for use in the proportion of 2 : 1.

**Technique.** Mix 1 to 2 drops of gastric contents with 1 to 2 drops of the reagent in a small evaporating dish and evaporate to dryness on a water bath. If free hydrochloric acid is present a reddish colour is produced. A positive test is only given by mineral acids, not by organic acids. Since hydrochloric acid is the only mineral acid likely to be present a positive test shows the presence of free hydrochloric acid.

### Tests for Lactic Acid

1. **Ferric Chloride Test.** This is the simplest test for lactic acid. To about 2 ml. of filtered gastric contents in a test tube add 1 to 3 drops of 10 per cent. ferric chloride solution. A yellow colour is given by lactic acid. Almost fill the tube with water and mix by inversion. The yellow colour due to lactic acid remains on dilution. Alternatively place about 3 drops of the ferric chloride into a test tube, fill about three-quarters full with water, mix, and add 1 to 2 ml. of gastric contents. Lactic acid gives a yellow colour.

2. **Ferric Chloride Test after Ether Extraction.** Extract about 5 ml. of filtered gastric contents with 20 ml. of ether. To about 5 ml. of clear ether extract add 20 ml. of distilled water and 2 drops of 10 per cent. ferric chloride solution. Shake. Lactic acid gives a yellowish-green colour.

3. **Maclean's Test.** To 5 ml. of filtered gastric contents add a few drops of Maclean's solution. This contains 100 ml. of saturated mercuric chloride solution and 1·5 ml. of hydrochloric acid, in which 5 grams of ferric chloride are dissolved. A reddish colour is given by lactic acid. Compare with a blank done on normal gastric contents.

4. **Uffelmann's Test.** *Uffelmann's Reagent.* Add 10 per cent. ferric chloride solution drop by drop to a 1 per cent. solution of phenol in water until a bluish colour is produced. It is essential to prepare immediately before use. Use distilled water.

Mix equal volumes of this reagent and of filtered gastric contents. A yellow or greenish colour is given by lactic acid.

Tests for lactic acid are not always very satisfactory, particularly when only small amounts are present.

For ordinary routine work no further investigations are required. However, determination of chlorides and examination for the presence of digestive enzymes are occasionally carried out.

### Determination of Chlorides

Chlorides present in gastric contents include free hydrochloric acid; a small amount of hydrochloric acid combined with protein—protein hydrochloride; and inorganic chlorides, mainly sodium chloride—

mineral chloride. Total chlorides– the sum of these three—and mineral chlorides are determined.

### 1. Total Chlorides

**Reagents.** These are those used for blood chlorides, p. 329.

1. N/20 silver nitrate in nitric acid.
2. N/50 ammonium thiocyanate.
3. Ferric alum, 5 per cent. aqueous solution.

**Technique.** Add 1 ml. of filtered gastric contents slowly to 3 ml. of the silver nitrate solution with constant shaking. Add a further 2 ml. of concentrated nitric acid and boil for one to two minutes. Cool, add 6 ml. of ferric alum solution, cool again, and back titrate with the thiocyanate. Check the standard by titrating 3 ml. of the silver nitrate with thiocyanate using ferric alum as indicator. This should require 7·5 ml.

**Calculation.** Ml. N/10 total chlorides per 100 ml. of gastric contents = (Titration of standard — titration of unknown) × 20.

This also gives the result in milliequivalents per litre.

### 2. Mineral Chlorides

These are determined in the same way after first evaporating off the hydrochloric acid. Pipette 1 ml. of filtered gastric contents into a small evaporating dish and evaporate to dryness on a water bath. Then heat carefully over a Bunsen burner until a greyish ash is obtained. Avoid overheating since some of the mineral chlorides may then be lost by volatilization. A red heat must not be reached. Add 3 ml. of the silver nitrate solution, 2 ml. of nitric acid, and continue as above. The calculation is similar.

Ml. N/10 mineral chlorides per 100 ml. of gastric contents
= (Titration of standard — titration of unknown) × 20.

#### INTERPRETATION

The total chloride content of gastric juice is about 155 milliequivalents per litre, but in the gastric contents as obtained in a test meal is lower than this due to admixture with varying amounts of other material. In resting contents it may range from about 40 to 150 milliequivalents per litre. The total chloride falls considerably after taking the meal, then rises as gastric secretion is produced.

The term *active acidity* has been given to the difference between the total and mineral chlorides. It thus corresponds to the free hydrochloric acid plus the protein hydrochloride.

The presence of considerable amounts of mineral chloride has been held to show that regurgitation from the duodenum has taken place with consequent neutralization of acid in the stomach.

## Tests for Digestive Enzymes

Tests for both pepsin and rennin have been used to show whether the stomach is secreting digestive enzymes.

**Rennin.** Bockus (1943) has used a simple method for testing for

the presence of gastric rennin. Add 5 drops of gastric juice to 5 ml. of fresh milk in a test tube and place in an incubator or water bath at 40° C. for fifteen minutes. If rennin is present, coagulation of the milk occurs.

**Pepsin.** An early method much used is that of Mett, in which narrow glass tubes are filled with coagulated egg white and placed in a mixture of gastric contents and hydrochloric acid and incubated for twenty-four hours at 37° C. The amount of digestion is related to the length of protein digested. The method is described fully by Hawk *et al.* (1947).

Hunt has published a convenient method which uses the digestion of dried serum.

### Determination of the Peptic Activity of Gastric Contents (Hunt, 1948)

Anson and Mirsky (1932) used carboxyhæmoglobin as substrate, and modifications of their method have been published by Beazell *et al.* (1938), and by Bueher *et al.* (1945). Hunt uses dried plasma or serum, but measures the peptic activity by the same method, utilizing the blue colour given with the Folin and Ciocalteu reagent.

**Reagents.** 1. Trichloracetic acid, 0·35 N solution. This is approximately 6 per cent. w/v. Standardize to 0·35 N.

2. N/4 sodium hydroxide.

3. Hydrochloric acid, *p*H 2·1.

4. Folin and Ciocalteu reagent (see p. 310).

5. Standard phenol solution. Five mg. per 100 ml. solution.

6. Substrate. Dissolve 5·6 grams of dried citrated plasma or dried serum in 100 ml. of distilled water with sufficient hydrochloric acid to give an acidity of *p*H 2·1 as determined by a glass electrode at 18° to 22° C. Stir the solution and filter through cotton wool.

**Technique.** For each determination place four tubes, (1) and (2) containing 5 ml. of substrate, and (3) and (4) 10 ml. of the trichloracetic acid, in the water bath at 37° C. Mix the gastric juice with an equal volume of hydrochloric acid of *p*H 2·1, warm to 37° C., and add 1 ml. of the mixture to each of tubes (1) and (4). Incubate for fifteen minutes, at the end of which time tube (1) is mixed with tube (3) and tube (2) with tube (4), by pouring backwards and forwards. Allow to stand in the bath for about four minutes. (1) + (3) gives the test, (2) + (4) the control. Filter. Fifteen to thirty minutes after the beginning of filtration, pipette 2 ml. of the filtrates into 50 ml. stoppered cylinders containing 20 ml. of N/4 sodium hydroxide. Mix by gentle rotation, and add 1 ml. of Folin and Ciocalteu reagent, and again mix by gentle rotation. Read the colour in thirty minutes, using a red filter or transmission at 680 millimicrons.

**Calculation.** The difference between the test and the control gives a measure of the peptic activity. As standard, mix 2 ml. of a freshly prepared phenol solution containing 5 mg. per 100 ml., with 20 ml. of N/4 sodium hydroxide, and add immediately 1 ml. of Folin and Ciocalteu reagent. Read in the colorimeter after five to ten minutes. Put up a blank omitting the phenol. A gastric juice is said to have

50 units of peptic activity per ml., when the colour produced in the above estimation equals that of this standard.

FRACTIONAL TEST MEAL. INTERPRETATION

## Resting Contents

**Volume.** In most normal cases after a night's fast only a small quantity (20 to 50 ml.) of resting contents is obtained. It is difficult to give any hard and fast dividing line above which the volume can be regarded as pathological, but anything above 100 to 120 ml. may be considered to be abnormal.

An *increased volume* of resting contents may be due to hypersecretion of gastric juice ; to retention of gastric contents, that is, to delayed emptying of the stomach ; and to regurgitation from the duodenum. When there is hypersecretion the contents are clear and almost colourless, and usually have a rather high free acidity. The increase in volume is often moderate and is found most commonly in patients who have a duodenal ulcer. With delayed emptying, the most important cause of increased resting contents, large quantities of food residues may be held back in the stomach. This occurs when there is pyloric obstruction, as may be the case in malignant disease of the stomach, when there is usually achlorhydria and frequently organic acids present, or in duodenal ulcer, when free acid is present often in higher concentration than in normal resting contents. Very large quantities, up to 1 to 2 pints, may be withdrawn. Tests for starch are positive, whereas normally they are negative. Microscopic examination shows the presence of food residues. Finally, if there is increased volume due to regurgitation from the duodenum, bile will be present. This is not an important cause of such an increase in the fasting state.

**Bile.** Bile may be found occasionally but is not usually of any particular significance. A small amount may be regurgitated from the duodenum into the stomach as a result of the nausea which some people experience in swallowing the tube.

**Blood.** Blood should not be present. A small amount of fresh bright red blood is occasionally seen in the resting contents and towards the end of the test when the stomach is almost empty. Such blood is due to trauma and as a rule is easily distinguished from blood arising from some pathological condition. Blood which has been in the stomach for some time is usually brown or reddish-brown in colour. In the presence of hydrochloric acid, red blood cells are hæmolyzed and dark brown acid hæmatin formed. This occurs in gastric ulcer, and occasionally in gastric carcinoma. When the presence of blood is associated with delayed emptying of the stomach, the blood is usually mixed with food giving various shades of brown according to the amount of blood present. This is sometimes referred to as a " coffee grounds " appearance. Blood mixed with food is most commonly found in gastric carcinoma. In addition, bleeding occasionally occurs in gastritis. It is necessary to remember the possibility that blood arising from a lesion of the upper or lower

respiratory passages may be swallowed and appear as altered blood in gastric contents.

**Organic Acids. Lactic Acid.** It is now generally held that lactic acid and other organic acids are formed by the action of bacteria on food remaining in the stomach. For such action to take place there must be little or no hydrochloric acid present and food must remain in the stomach for rather long periods of time. Achlorhydria associated with retention of food thus gives the most favourable conditions for this fermentation, so that lactic acid is found almost exclusively in gastric carcinoma. The view held at one time that lactic acid could be secreted by the stomach in this condition has been abandoned.

**Mucus.** Normally mucus is only present in small amounts. It is increased in gastric carcinoma and in gastritis. Generally speaking the quantity is inversely proportional to the amount of hydrochloric acid present. Much mucus in the resting contents is often due to swallowed saliva.

**Residual Contents.** The stomach may be found to be empty during the last hour of the test in normal people. Otherwise not more than 30 ml. of residual contents remain at the end of two and a half hours. The most common cause of an increased amount is delayed emptying of the stomach. Then several hundred ml. of residual contents may be withdrawn.

## Acidity Curves

### 1. Normal Findings

There is usually a small amount of free hydrochloric acid in the resting contents. This is almost always below 30 units (in ml. N/10 acid per 100 ml. of gastric contents). The total acidity lies about 8 to 10 units higher. An appreciable number of cases show no free acid in the resting contents. After drinking the meal there is a short period, from a quarter to three-quarters of an hour in most cases, before free acid is found again. The free acid then rises steadily to reach a maximum at about one and a quarter to one and a half hours, after which the concentration of free acid begins to decrease. The free acid ranges from 15 to 45 units at the maximum, with the total acid about 10 units higher. A typical normal curve is shown in Fig. 41. About 80 per cent. of normal people fall within these limits. A few per cent. of apparently normal persons exceed the higher of these values whilst the rest are below the figures given. A complete absence of hydrochloric acid may also be found in apparently normal persons. Whereas this is very rare in young children, it is found in about 4 per cent. of young adults and rises steadily with increasing age. At sixty to seventy years it is found in some 20 per cent. of persons, but does not increase further with age.

The stomach may be found to be empty at any time during the last hour of the test. Blood should not be present and there should not be any appreciable amount of bile. Some may be found in the resting contents (see p. 205) and traces may be present towards the end of the

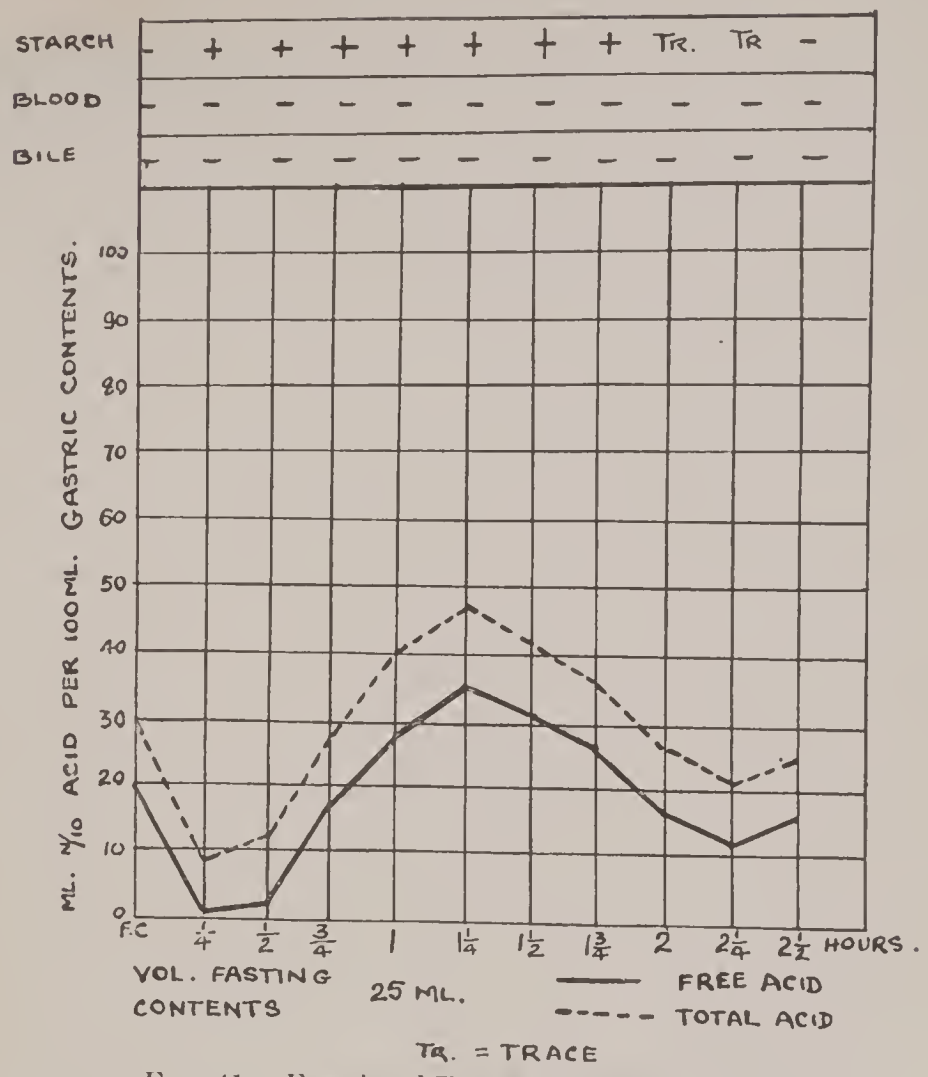


FIG. 41. Fractional Test Meal. Normal Result.

test, but heavily stained specimens obtained late in the test are most frequently due to the tube passing into the duodenum (see p. 199). In the case of patients following partial gastrectomy or after gastro-enterostomy, mixture of the stomach contents with bile is usual and largely unavoidable. In these patients the value of the fractional test meal is doubtful because of technical difficulties in siting the tube and avoiding regurgitation of duodenal contents.

## 2. Abnormal Curves

The two most important groups showing abnormal results are those with *hyperchlorhydria* (hyperacidity) in which the free acid reaches a higher concentration than in normal persons, and those with *achlorhydria* in which there is no secretion of free acid. A region of *hypochlorhydria* (hypoacidity) in which the acid, though present, is in a concentration below the normal range, is sometimes recognized though the region is small. In any case most of the patients showing hypochlorhydria belong to conditions in which there is an increased incidence of achlorhydria. Many such patients subsequently develop achlorhydria.

**Hyperchlorhydria.** This may be said to occur when the maximum free acidity exceeds 45 units. Various figures have been suggested for this dividing line. These range from 40 to 60 units, and several workers prefer to take 50 units. As with most limits of normal there is no hard and fast dividing line. The acidity refers to the concentration of hydrochloric acid in the gastric contents, not to the acid content of the gastric juice as secreted. The acid is thought always

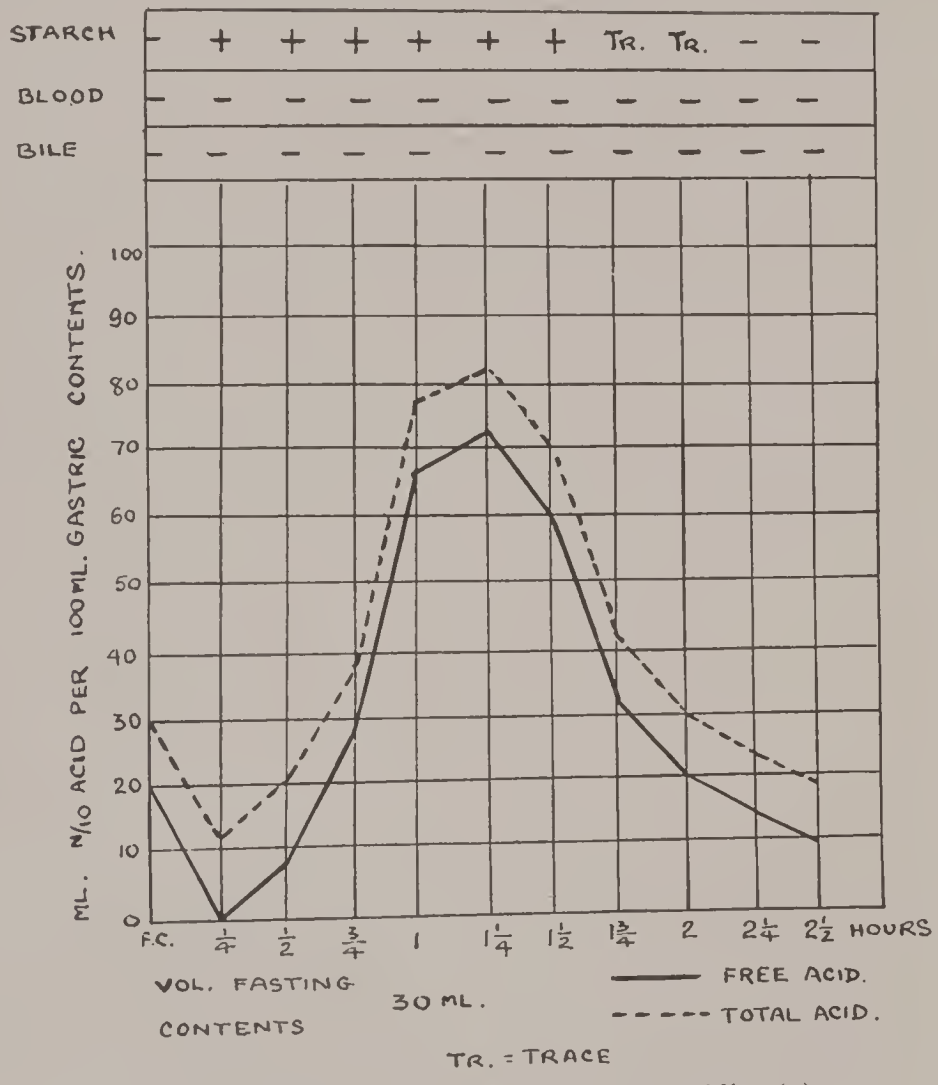
STARCH
BLOOD
BILE
+ + + + + + TR. TR. - -
- - - - - - - - - - -
- - - - - - - - - - -
ML. N/10 ACID PER 100 ML. GASTRIC CONTENTS.
0 10 20 30 40 50 60 70 80 90 100
F.C. ¼ ½ ¾ 1 1¼ 1½ 1¾ 2 2¼ 2½ HOURS
VOL. FASTING CONTENTS
30 ML.
FREE ACID.
TOTAL ACID.
TR. = TRACE

Fig. 42. Fractional Test Meal. Hyperacidity (1).

to be secreted at the same concentration though the volume of secretion may be increased.

Types of curve which may be obtained in hyperchlorhydria are shown in Figs. 42 and 43. Curves intermediate between these may also be seen. With the type (Fig. 42) the rate of emptying of the stomach may be normal or even more rapid than usual, whereas the type illustrated in Fig. 43 is mostly obtained in patients with some abnormality of the pylorus so that there is delayed emptying. The fact that there is a small incidence of hyperchlorhydria in apparently normal people should always be borne in mind.

At least 70 per cent. of cases of *duodenal ulcer* show hyperchlor-

hydria, a much greater percentage than that found in any other condition. The remaining cases are almost all within normal limits. In those patients with hyperacidity the acidity of the fasting contents is usually increased and after the fall in acidity when the meal is taken, there is a rapid rise in the concentration of acid to levels above normal. In some cases very high values exceeding 100 units, and even reaching 130 units, are found for the free acid. Although they are seen occasionally in patients with gastric ulcer, curves similar to that shown in Fig. 43 are most characteristic of duodenal ulcer, since

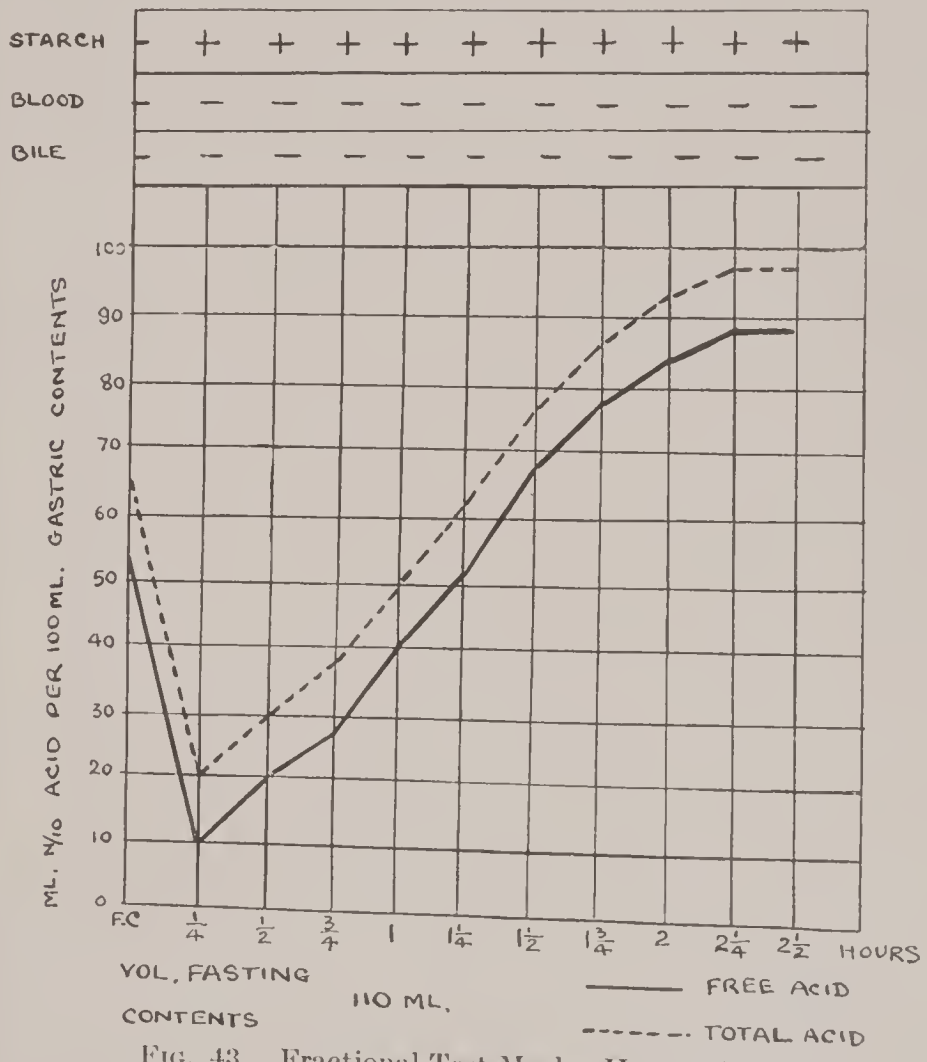


FIG. 43. Fractional Test Meal. Hyperacidity (2).

involvement of the pylorus is more common. This type of curve is often termed the climbing curve because the concentration of free acid climbs steadily to high levels and shows little or no sign of falling by the time the end of the test is reached. Where there is pyloric stenosis the volume of resting contents and of residual contents may be very high indeed. From 1 to 2 pints of resting contents may be withdrawn and up to 300 to 400 ml. residual contents. Charcoal will be present in the resting contents if it was given, and a positive test for starch will show the presence of food residues. In absence of pyloric stenosis or spasm some increase in resting contents may be

found but will be moderate, usually not more than 100 to 200 ml. Bockus stresses the value in duodenal ulcer of strongly positive tests for blood found only when bile is present.

In *gastric ulcer* the findings are not so characteristic. Though there is an increased incidence of hyperchlorhydria, normal results are obtained in approximately 50 per cent. of cases, whilst in some chronic cases a hypoacidity or achlorhydria may develop as a result of an associated gastritis. Blood may be present, particularly in the resting contents. Blood together with hyperchlorhydria is suggestive of gastric ulcer, but may also be found occasionally in gastric carcinoma. An ulcer in the stomach may also give rise sometimes to pyloric stenosis or spasm.

*Jejunal and gastrojejunal ulcers* occur as sequelæ to gastro-enterostomy. They are more often found when the hyperacidity persists after the operation.

It should also be noted that a small percentage of cases of *cancer of the stomach* are found to have hyperchlorhydria.

**Achlorhydria.** This term is used when there is no secretion of hydrochloric acid. Enzymes such as pepsin may still be present in the gastric juice. When both enzymes and acid are absent, the term *achylia gastrica* is used to show there is a complete absence of gastric secretion.

As stated earlier, achlorhydria is found in some apparently normal people, increasing with age to about sixty to sixty-five years old. There is an increased incidence in illness generally. In particular this is the case in wasting and febrile diseases, being part of the general weakness, examples being tuberculosis, Addison's disease, the later stages of malignant conditions, ulcerative colitis, and fevers. An increased incidence has been reported in certain skin diseases such as rosacea, and in some allergic conditions such as asthma and urticaria, but the relation of several of these diseases to achlorhydria is uncertain, particularly if the known incidence of achlorhydria for a particular age group is borne in mind.

It is clear, however, that in microcytic anæmia due to iron deficiency, there is a considerable increase in the incidence of achlorhydria, which has been said to be present in 80 per cent. of cases (Wintrobe, 1947). There is also an appreciable increase in hyperthyroidism and in myxœdema (Lermon and Means, 1932).

Patients who have achlorhydria sometimes have a looseness of the stools, a condition known as enterogenous diarrhœa. Such attacks of flatulence and diarrhœa often benefit from taking acid and pepsin.

As regards diseases of the stomach there is a high incidence of achlorhydria in *cancer of the stomach*. Almost half the cases may be expected to show it when first examined and half the rest to show hypochlorhydria. The examination of the resting contents is of special importance, particularly if there is some pyloric obstruction. The volume is then raised, sometimes very considerably, to several 100 ml. and food residues intermixed with blood are present. Lactic acid can frequently be detected. Altogether the resting contents present a very typical picture in such cases. The combined acid is greatly

increased, usually ranging from 30 to 80 units, though it may be higher. The Günzberg test for free hydrochloric acid is negative. When it is possible to remove the mass of resting contents almost completely, the type of curve obtained is that shown in Fig. 44. Often, however, it is difficult, if not impossible, to remove the mass of material present through the narrow stomach tube used. As a result, after the meal has been drunk, subsequent specimens are still found to contain a certain amount of resting contents, so that the total

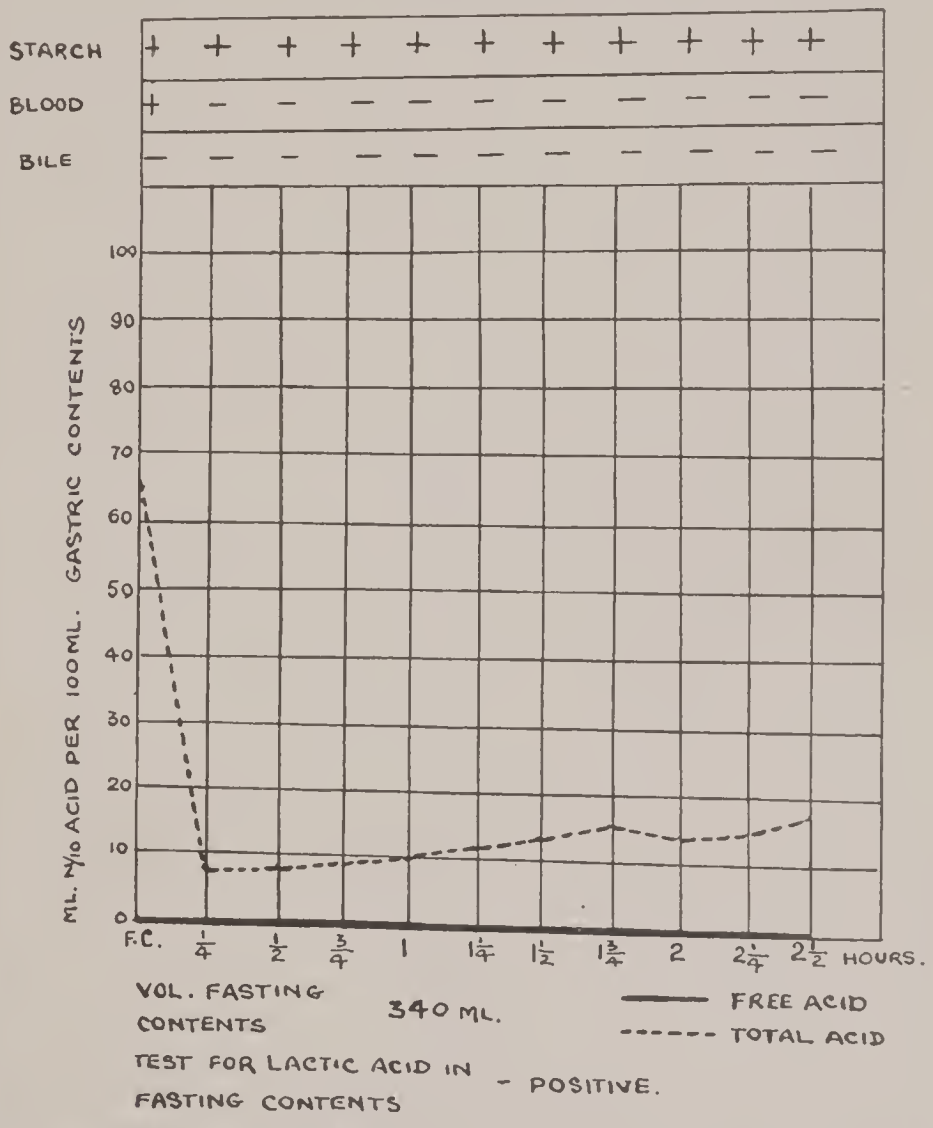


Fig. 44. Fractional Test Meal. Achlorhydria, Carcinoma Stomach.

acidity throughout the test is still higher than usual and blood and lactic acid are present in most of the specimens. It is only in advanced cases of cancer of the stomach that a true achylia gastrica is present.

In *chronic gastritis*, though all types of curve may be obtained, the tendency is for gastric acidity to be reduced. As the disease progresses there is a gradual loss of acid secretion and an increasing occurrence of achlorhydria, which may ultimately become an achylia gastrica.

Whilst *gastroenterostomy* almost never leads to true achlorhydria

when carried out in patients with duodenal ulcer, gastric acidity is often reduced since regurgitation of bile is frequent and emptying of the stomach rapid. *Partial gastrectomy*, however, does often lead to a reduction in gastric acidity and to achlorhydria in a considerable number of cases.

Whilst it is only in the most advanced cases of cancer and gastritis that an achylia develops, there is achylia gastrica in 100 per cent. of cases of *pernicious anæmia* and of *subacute combined degeneration of the spinal cord*. There is usually little or no resting contents and the

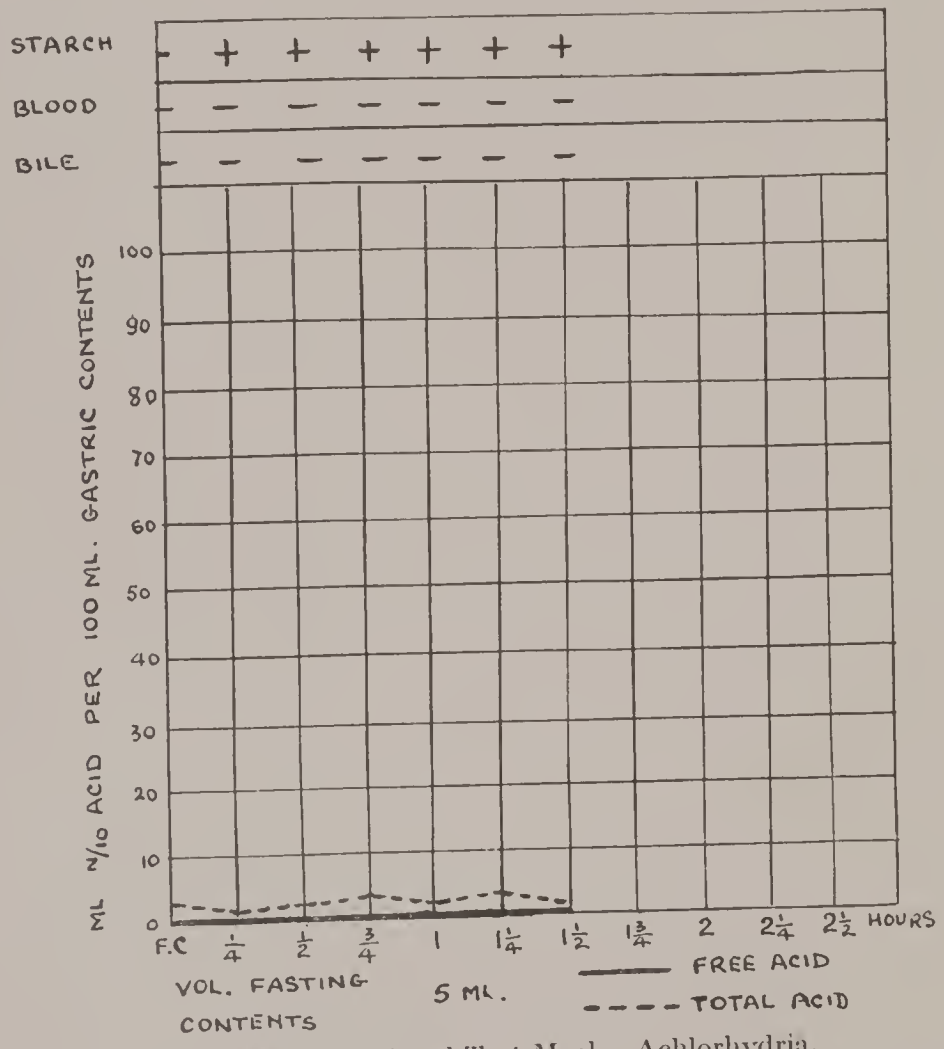


Fig. 45. Fractional Test Meal. Achlorhydria.

stomach tends to empty rapidly so that it may be empty any time from an hour after taking the gruel. Blood and bile are absent as are organic acids. Total acidity is small, often only about 4 to 6 units. An example is shown in Fig. 45.

While no hard and fast line can be drawn, emptying is not usually as rapid in cases of simple achlorhydria as when there is achylia. In many cases the full range of specimens up to the end of two and a half hours can be obtained, starch may be present throughout and total acidity is more often in the region of 10 units (Fig. 46).

It is clear that achlorhydria often arises as a secondary manifesta-

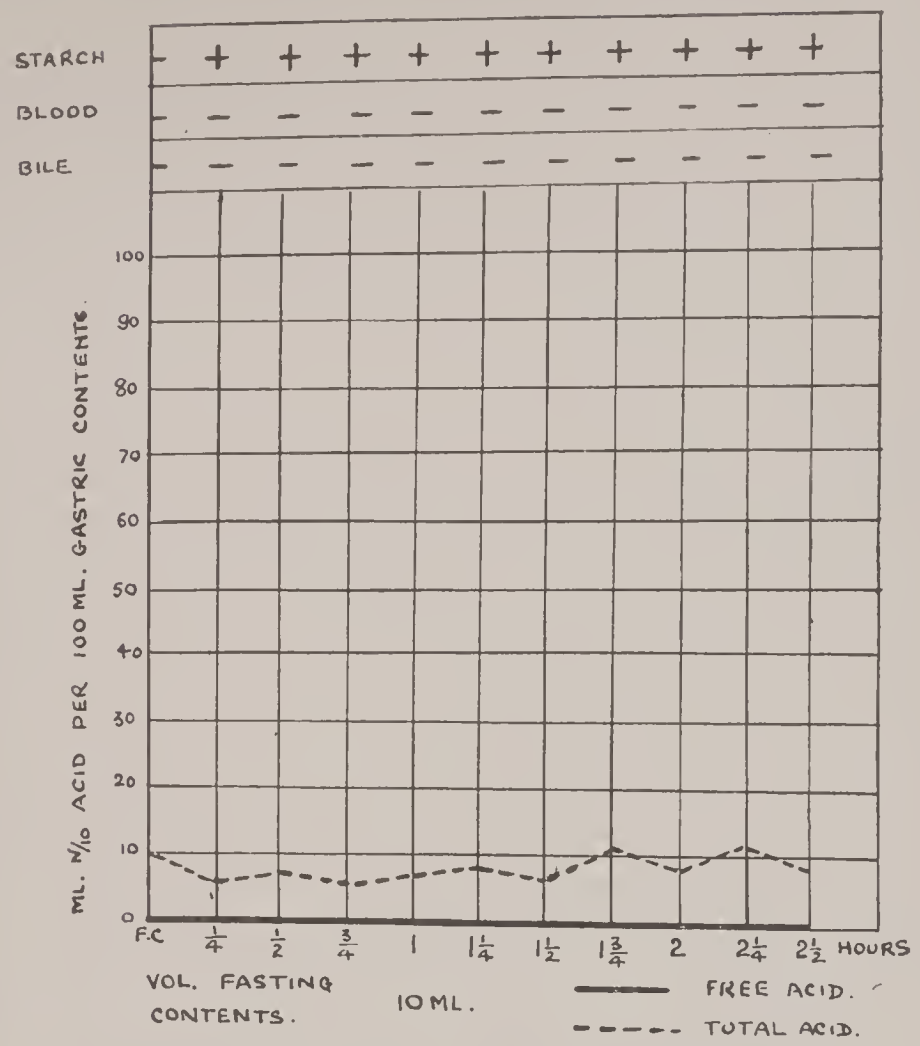


Fig. 46. Fractional Test Meal. Achlorhydria.

tion. Nevertheless, impaired absorption of minerals such as iron, and of vitamins, as a result of the achlorhydria may be of importance in the ætiology of some of the diseases in which it is found. The loss of the action of the acid on bacteria in the stomach may also be important in connection with some cases of diarrhœa and colitis.

## Summary

It is seen that whilst the fractional test meal is of limited value as a definite aid in the diagnosis of the individual with dyspepsia, if it is used with discretion in conjunction with clinical findings it may give useful information in regard to the concentration of acid in the stomach and as to the state of the pylorus. It is valuable also in revealing the existence of achlorhydria. Further information as regards the type of achlorhydria, which is particularly useful in pernicious anæmia and subacute combined degeneration of the spinal cord, is given by use of histamine in these cases.

## THE USE OF HISTAMINE

Histamine is a powerful stimulant for the secretion of hydrochloric acid in the normal stomach. Collection of gastric contents after its

injection is used in investigating cases of achlorhydria revealed by the fractional test meal. The so-called **Histamine test meal** can be carried out as follows. Pass a Rehfuss or Ryle tube as for the fractional test meal and withdraw the resting contents. Inject 0·25 mg. of histamine hydrochloride subcutaneously. Larger amounts have been used, up to 0·01 mg. per kilogram of body weight, but rather unpleasant reactions may be encountered with such doses. After the injection remove 10 ml. of stomach contents every ten minutes up to the end of an hour so far as this is possible. Filter the specimens and titrate and examine as described above for the fractional test meal.

A number of cases which show achlorhydria in response to the fractional test meal are found to secrete acid when given histamine. On the other hand histamine is not able to cause any secretion of acid in patients with achylia gastrica. Cases which do not produce acid after histamine injection have been described as histamine resistant achlorhydria. The demonstration that such an achlorhydria is present may be useful in the diagnosis of subacute combined degeneration of the spinal cord and pernicious anæmia. For such a purpose the test may be done instead of the fractional test meal.

Much higher concentrations of acid are found in the specimens withdrawn in the histamine test than in the fractional test meal. Specimens containing 50 to 100 units of free acid are frequently obtained in normal patients. When hyperchlorhydria has been found in the fractional analysis, for example in duodenal ulcer, more juice may be secreted and a higher concentration of acid found in the specimens obtained after giving histamine than in normal cases. However, the test does not give any information in such cases which cannot be obtained by means of the ordinary test meal.

Histamine has been used in combination with the fractional test meal. If no free acid is found by the end of an hour and a half in the fractional test meal, histamine is given to see whether the stomach can produce acid in response to this more vigorous stimulus.

Histamine acts only on the acid secreting cells of the gastric mucous membrane. It is not a good stimulus for enzymes such as pepsin. Any increase in pepsin which results from injecting histamine is probably due to washing out enzyme already produced.

## THE ALCOHOL TEST MEAL

Alcohol has also been used as a stimulant for the secretion of acid. To carry out the test pass a Rehfuss or Ryle tube and remove the resting contents. Give 100 ml. of 7 per cent. alcohol and take samples of the stomach contents every fifteen minutes for about an hour and a half. Some methylene blue can be added to give an indication of the emptying time of the stomach. The specimens obtained are more easily pipetted without filtering than are those from the meal test and the alcohol is more easily drunk than the oatmeal gruel. The response to alcohol is more rapid and the stomach empties more quickly. Rather higher levels of free acidity

are obtained and the limits of normal are wider. Otherwise the findings in abnormal cases tend to parallel those already described for the gruel meal. The stimulus, whilst not so strictly physiological as that of the oatmeal, is rather more vigorous.

Histamine can also be combined with this alcohol test. If no free acid has been detected up to the end of an hour after giving the alcohol, an injection of histamine can be given as previously described, to see if acid is then produced.

## INSULIN "TEST" MEALS

When vagal resection is carried out as a treatment for duodenal ulcer an insulin "test meal" is used to determine whether the section of the vagus has been successfully performed. There is good evidence to show that the gastric secretion which results from the injection of insulin is due to the central stimulation of the vagus by the hypoglycæmia. For this effect to occur, a significantly low blood sugar

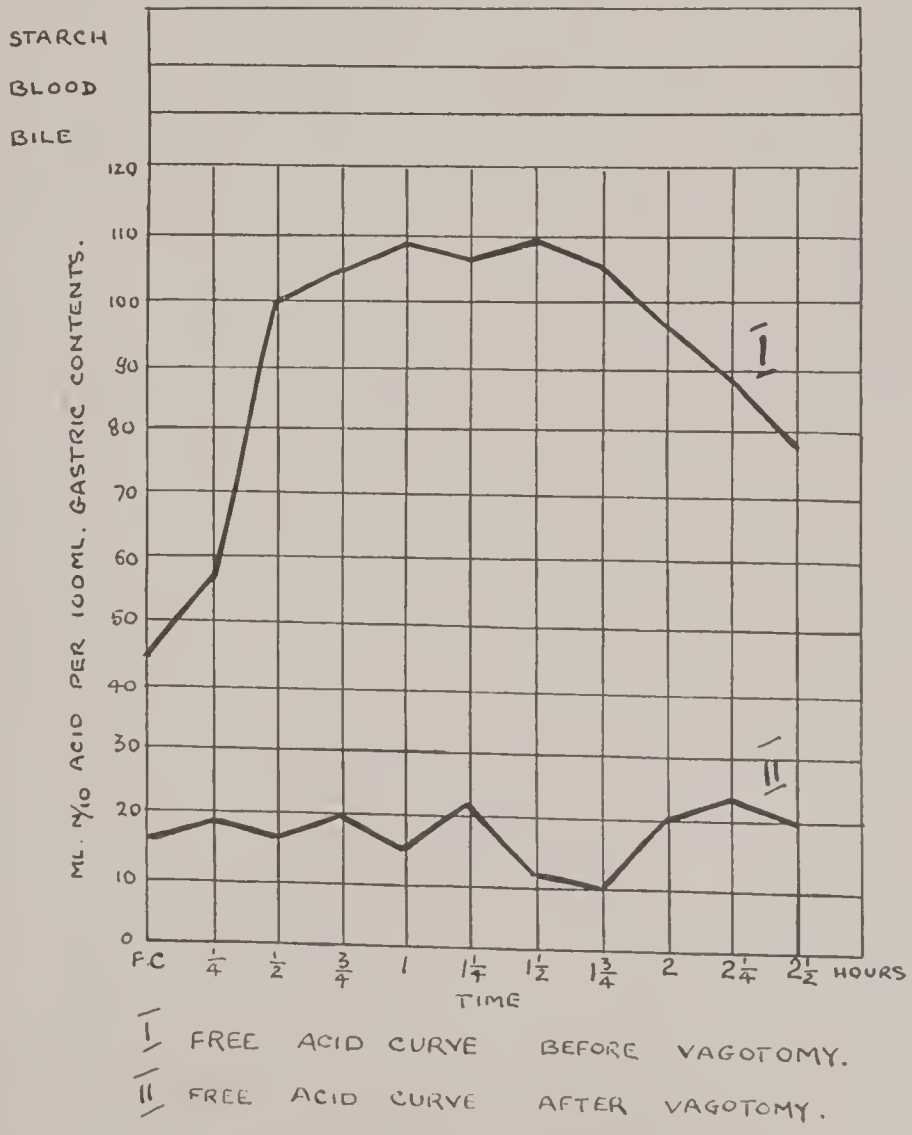


FIG. 47. Insulin Test Meal.

must be produced. Hollander (1946, 1948, 1951) suggested that the blood sugar must be brought below 50 mg. per 100 ml. Brooke (1949) states that a level below 45 mg. per 100 ml. is necessary for a completely reliable test.

**Technique.** Carry out the test in the morning after a night's fast and give no breakfast. Pass a Rehfuss or Ryle tube and empty the stomach. Then give 15 units of insulin intravenously. This is the amount which has usually been given, but Brooke suggests that 10 units is sufficient. Patients rarely develop symptoms sufficiently severe or prolonged to require treatment with glucose. After injecting the insulin withdraw approximately 10 ml. samples of gastric contents every quarter hour for two and a half hours. Filter the specimens and examine as in the fractional test meal, titrating the free and total acid and testing for starch, blood, and bile. No starch should be present. Determine the blood sugar at least once, half an hour after giving the insulin, in order to make sure that a sufficiently low level is reached. If the blood sugar then is above 50 mg. per 100 ml. repeat the blood sugar estimation as soon as possible, but not later than an hour after giving the insulin.

**Results.** In patients before operation there is a marked and prolonged output of acid in response to the insulin. The concentration of free acid may rise to well over 100 units. After a successful vagotomy there is no response to the insulin. Typical pre-operative and post-operative findings are shown in Fig. 47.

It has been the practice of some surgeons to have the test done pre-operatively, then soon after the operation and once again several months later. Brooke has suggested that it is sufficient and quite satisfactory only to do the test once, at least six months after the operation. He also emphasizes that although lack of an acid secretion in post-vagotomy patients, when the blood sugar has been reduced to below 45 mg. per 100 ml., cannot be regarded as certain evidence that all the fibres of the nerve have been cut, it can usually be presumed that this is the case.

## TESTS FOR BLOOD IN FÆCES

Examination of the fæces for the presence of altered blood is important in the diagnosis and treatment of organic lesions of the alimentary tract. Several tests are available but the benzidine test in one form or another is most commonly used.

### The Benzidine Test

Iron containing derivatives of hæmoglobin catalyse the oxidation of benzidine by hydrogen peroxide. Peroxidases in the fæces have a similar action, but are destroyed by heat before carrying out the test. Blue-coloured compounds are produced.

**Reagents.** 1. Benzidine, 10 per cent. solution in glacial acetic acid. Prepare freshly every two to three days, and keep in the dark when not in use.

2. Hydrogen peroxide, 10 volumes per cent. Prepare at frequent

intervals from stock 100 volumes per cent. by diluting 1 to 10 with water.

**Technique.** Take a small portion of fæces about the size of a large marble and mix thoroughly with about 10 ml. of water in a 6 × 1 inch test tube. Boil for about a minute in order to destroy peroxidases. Cool. Mix equal volumes of the benzidine solution and the hydrogen peroxide in another test tube. After making sure that no colour develops in this mixture (about one minute) add fæcal suspension slowly with shaking until an amount equal in volume to the mixture of benzidine and peroxide has been added. The test is positive if a blue or greenish-blue colour is produced. A rough indication of the amount of blood present can be obtained by observing the speed with which the colour develops, and the intensity of the colour produced. All types of response from a deep blue colour with one or two drops of suspension to a negative result are obtained. Tests can be reported strongly positive when a deep blue colour develops immediately on adding the first few drops of fæcal suspension, positive when the colour develops immediately on mixing and deepens rapidly, weakly positive when the colour develops only slowly, and negative when there is no blue colour.

The test tubes used should be thoroughly cleaned out with soap and water, well rinsed with water, and allowed to stand with distilled water in them until used.

**Notes.** 1. *Preparation of the Patient.* Before the test is done it is essential that the patient should have been on a hæmoglobin-free diet for at least two to three days. Meat and meat products are forbidden during that time. Some workers also exclude chlorophyll by omitting green vegetables. A check on the adequacy of the dietary restrictions can be made by examining the fæces microscopically for partially digested muscle fibres if a positive test is obtained (see p. 280). If these are present a further specimen of fæces should be collected. As regards iron salts taken as treatment for anæmia, while weakly positive tests have been found sometimes in the apparent absence of blood if iron is being taken, other workers have reported iron salts to have no effect on the test (Hughes, 1952).

2. The benzidine test as described above is a very sensitive test. On microscopic examination the fæces of persons without any apparent lesion of the gastro-intestinal tract are often found to contain small numbers of red blood cells. It has been felt by some workers, therefore, that the test as ordinarily carried out is too sensitive. Sensitivity can be diminished by using less concentrated solutions of benzidine. King (1951) uses 3 per cent. benzidine in glacial acetic acid instead of the 10 per cent. solution used above, which approximates to the saturated solution used by a number of workers (for example, Harrison, 1947), in which case a knife point of benzidine is shaken with a few ml. of acetic acid, an equal volume of hydrogen peroxide added and the test done as described above. This latter technique gives results similar to those using 10 per cent. benzidine solution. Using a 3 per cent. solution a number of tests are found to be negative, which give positive results with 10 per cent.

or saturated solutions of benzidine. More information is obtained if several concentrations of benzidine, for example, 1, 3, 5 and 10 per cent., are used. Positive results with 1 and 3 per cent. may be taken to indicate the presence of bleeding with a high degree of certainty, particularly with the 1 per cent., but negative results do not completely exclude it. Positive results with 5 and 10 per cent. benzidine, especially the latter, are of doubtful significance.

3. The question of varying the sensitivity of the technique used has also been related to the effect of taking meat. Many workers have wished to avoid the trouble of placing the patient on a meat-free diet for several days. By reducing the sensitivity of the test, it has been hoped a technique could be developed which would obviate the need for such dietary restrictions. The author at one time investigated the use of a wide range of concentrations of benzidine in this connection. It appeared that if a positive test was obtained using a 1 per cent. solution of benzidine, this could be taken to indicate the presence of bleeding even without special dieting.

Gregerson used a slide test with the same object. The fæces are not boiled in this test, but a small portion of fæces, best taken from inside the stool, is smeared on a slide. A mixture of 0·2 gram of barium peroxide and 0·025 gram of benzidine is dissolved in 5 ml. of 50 per cent. acetic acid, and a few drops of this added to the fæces on the slide. As before, a green or blue colour indicates a positive result. If this develops within thirty seconds it is said to indicate the presence of occult blood. Any colour produced after a minute is ignored. It is claimed that apart from excluding such foods as black puddings, and liver, dietary restrictions are not required (see Ogilvie, 1927 ; Lipetz, 1950), but it is stated that while a positive result is always significant, a negative one is not conclusive. The position with the test as described above, using 10 per cent. or saturated benzidine solutions, is the reverse. Then a series of negative results may be taken to exclude bleeding with a high degree of certainty ; it is only weakly positive tests which may be of doubtful significance.

Recently Needham and Simpson (1952) have used a modified Gregerson test which is carried out on a white filter paper instead of on a slide so that the blue colour can be more easily seen. Glacial acetic acid is used to dissolve each powder, a number of which are prepared and kept wrapped in waxed paper. Results are reported positive when a deep blue colour appears within fifteen seconds, weakly positive if a greenish blue colour is seen within thirty seconds, and negative if no colour is obtained within thirty seconds. These authors also studied the effect of giving known amounts of blood to patients free from bleeding. They consider the test in this form to have advantages over other methods both for in-patients and out-patients.

See also Hughes (1952).

### The Guaiac Test

**Reagents.** 1. An alcoholic solution of gum guaiacum. A solution containing 1 gram per 100 ml. of 95 per cent. alcohol is recommended. Prepare freshly.

2. Hydrogen peroxide. Ten volumes per cent.

**Technique.** A small portion of fæces is mixed with a few ml. of water and boiled as before to inactivate enzymes. After cooling add to about an equal volume of a 1 : 1 mixture of the alcoholic gum guaiacum and hydrogen peroxide. A positive test is shown by the development of a blue colour.

This test is not nearly so sensitive as the benzidine test. Appreciable amounts of blood may not be detected. Whilst this may sometimes be due to the use of unsatisfactory gum guaiacum this is not always the case. The test is not recommended.

### The Reduced Phenolphthalein Test

**Reagents.** 1. Reduced phenolphthalein. Dissolve 2 grams of phenolphthalein and 25 grams of potassium hydroxide in 100 ml. of water, and add 10 grams of powdered zinc. Mix well, and warm gently until the red colour disappears completely. Filter, and add a little zinc to the filtrate to prevent oxidation.

2. Glacial acetic acid.

3. Hydrogen peroxide, 10 volumes per cent.

4. Ether.

**Technique.** Make a small amount of fæcal suspension in water, and add a little glacial acetic acid. Extract with 10 to 20 ml. of ether and pour this extract into a test tube. Add about 1 ml. of reduced phenolphthalein, shake gently, and add a few drops of hydrogen peroxide. A pink to red colour is produced if blood is present, fading rather quickly with small amounts, but persisting longer according to the amount of blood present. The intensity of the colour is roughly proportional to the amount of blood present. The colour may be given if hydrogen peroxide is not added, but the test is then less sensitive. The ether extract must not be too acid, and if much acetic acid has been added it is best to neutralize by adding a little 10 per cent. sodium hydroxide.

The test appears to be a little less sensitive than the benzidine test as described above, but appreciably more sensitive than the guaiac test.

#### INTERPRETATION

A positive test indicates that blood has been introduced at some point between the mouth and the anus. If the test is being used in the diagnosis and treatment of gastric or intestinal diseases it is obviously necessary to exclude the possibility that blood has been introduced higher up or lower down the alimentary tract. The effect of hæmorrhoids is particularly important. In such cases the blood can sometimes be seen as streaks of fresh blood on the surface of the stool. Inspection for such streaks should always be made. If any doubt is felt whether such a streak is really blood, it should be examined microscopically. Naturally, in the presence of such blood further tests are valueless. Attention should also be paid to the possibility that blood from the mouth, nose, or throat may be introduced. That contamination with menstrual blood may occur should

also be borne in mind. Since there may be a little bleeding when a tube is passed for a fractional test meal false positive results may be obtained during the following two to three days.

The examination is mainly used in the diagnosis and treatment of ulcers and malignant growths of the stomach, duodenum, small and large intestine. It does not afford any information as to the type of lesion present. Bleeding may be intermittent so that the test is often carried out at intervals. It is also a useful practice to do the test on three successive days, when, if a patient is on a meat-free diet, this does usually ensure that by the time the third specimen is taken this is completely reliable. The test may give useful information in regard to the cessation of bleeding in the case of known ulcers, and so be used in deciding the rate of return to more normal diets.

When bleeding is severe the stools are dark coloured (melæna), but in other cases it does not affect the appearance of the fæces. The term *occult blood* is used to describe the blood present in such cases. In the course of its passage through the intestine oxyhæmoglobin is converted into hæmatin and porphyrins only the first of which gives positive tests. In the case of bleeding low down in the alimentary tract, however, oxyhæmoglobin may still be present. It can be recognized by spectroscopic examination of the supernatant fluid from a centrifuged fæcal suspension.

In addition to ulcers and malignant growths positive tests may be obtained in gastritis, and in hæmorrhagic diseases such as purpura and in scurvy.

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*CHAPTER XIV*

# TESTS IN LIVER AND BILIARY TRACT DISEASE

Tests used in the study of patients with liver and biliary tract diseases can be classified according to the specific function of the liver involved. These include :

1. Abnormalities of pigment metabolism.
   (*a*) Serum bilirubin and Van den Bergh reaction. Icteric index.
   (*b*) Urine bilirubin.
   (*c*) Urine and fæcal urobilinogen.
2. Tests based on the liver's part in carbohydrate metabolism.
   (*a*) Galactose tolerance test.
   (*b*) Lævulose tolerance test.
3. Tests based on changes in plasma proteins.
   (*a*) Determination of the plasma proteins—albumin, globulin and fibrinogen.
   (*b*) Flocculation tests. Thymol turbidity test and thymol flocculation test ; serum colloidal gold test ; cephalin cholesterol flocculation test ; zinc sulphate test ; Weltmann test ; Takata-Ara test ; formol gel test.
   (*c*) Amino acids in blood and urine (see pp. 155, 159).
4. Tests based on abnormalities of lipids.
   (*a*) Determination of serum cholesterol and of the proportion of free and ester cholesterol (see pp. 188–90).
   (*b*) Determination of fæcal fats (see p. 289).
5. Tests based on the detoxicating function of the liver.
   (*a*) Hippuric acid synthesis test.
6. Excretion of injected substances by the liver.
   (*a*) Bromsulphthalein test.
   (*b*) Bilirubin excretion test.
7. Formation of prothrombin. Determination of prothrombin time.
8. Miscellaneous.
   (*a*) Determination of serum alkaline phosphatase.
   (*b*) Determination of serum choline esterase.

Although this list includes the tests most frequently used in liver disease many others have been suggested and tried. See, for example, Lichtmann (1949).

## TESTS BASED ON ABNORMALITIES OF BILE PIGMENT METABOLISM. JAUNDICE

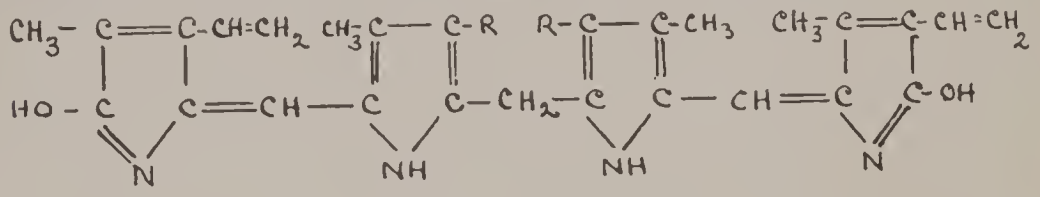
Since jaundice is due to an increase in the concentration of bilirubin in the blood, it is often, though not always, connected with liver and biliary tract disease. It will be most convenient to begin with a consideration of bilirubin and some substances derived from it. Of these we shall be almost solely concerned with bilirubin, urobilin and

urobilinogen. Their physiology and chemistry will be briefly summarized first.

Bilirubin is formed from hæmoglobin in the reticulo-endothelial system (spleen, bone marrow, Kupffer's cells in the liver), and then circulates in very low concentration in the blood. This bilirubin has been termed "blood bilirubin" or hæmobilirubin. It is excreted into the bile by the liver cells, when it undergoes some change, possibly in its linkage to protein. This bilirubin may be termed "bile bilirubin" or cholebilirubin. It passes in the bile into the intestine where it is reduced by bacterial action to urobilinogen. This is dealt with in two ways. The greater part is excreted in the fæces. There is then partial reoxidation on exposure to air, with formation of urobilin (stercobilin) and some darkening in colour. The rest of the urobilinogen is absorbed in the intestine and passes in the portal circulation to the liver. The greater part of this is re-excreted by the liver in the bile as urobilinogen or bilirubin. A small part, however, enters the general circulation and is excreted in the urine as urine urobilinogen. Again, some may oxidize to urobilin when the urine is exposed to air.

Chemically these substances contain the chain of four pyrrol groups obtained when the ring of these in the porphyrins (see p. 406) is opened.

*Bilirubin*, $C_{33}H_{36}O_6N_4$, has the structural formula :

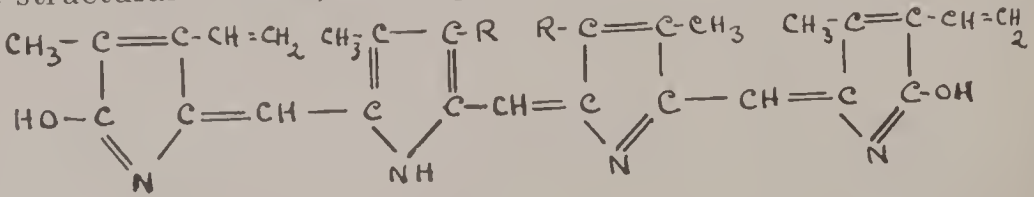


Bilirubin

in which R = $CH_2CH_2COOH$.

Bilirubin is obtained from evaporated bile or powdered gall stones when these are extracted successively with ether, hot water, 10 per cent. acetic acid, alcohol, and hot glacial acetic acid. These remove sterols, bile salts, biliverdin, and inorganic salts. The residue is dried and extracted with hot chloroform, from which the bilirubin can be crystallized. Bilirubin is insoluble in water, dilute acids, and the common fat solvents, but dissolves in hot chloroform and alkalies. Solutions of bilirubin do not show a characteristic spectrum.

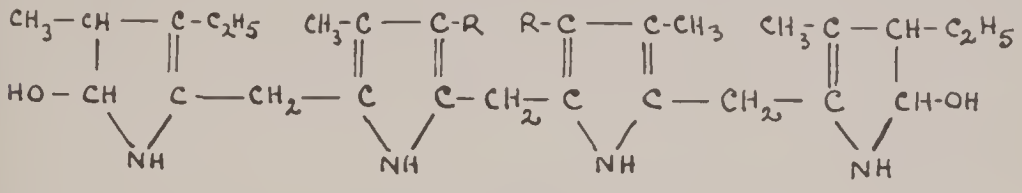
*Biliverdin*, $C_{33}H_{34}O_6N_4$, is formed by mild oxidation of bilirubin. Its structural formula, R being the same as in bilirubin, is



Biliverdin

It is formed spontaneously when bilirubin is oxidized by exposure to air in alkaline solution, by oxidation with ferric chloride in acetic acid, or is treated with hydrogen peroxide. It is precipitated on acidification with hydrochloric acid as floccules of hydrochloride. Unchanged bilirubin can be removed by chloroform extraction. Biliverdin is dark green, insoluble in water, moderately soluble in ether and chloroform, readily soluble in alcohol and glacial acetic acid. In alkalies it dissolves to form salts. These solutions are bright green and have an absorption band in the red.

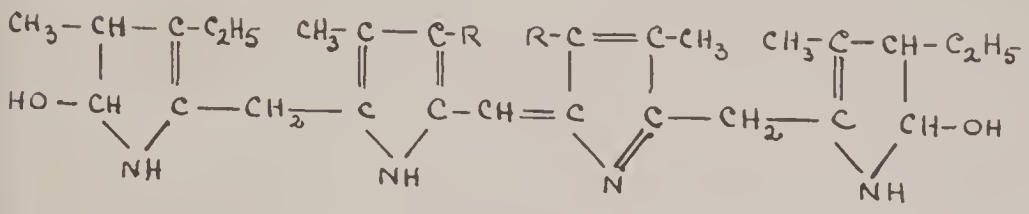
*Urobilinogen*, $C_{33}H_{48}O_6N_4$, has the structural formula (R as before):



Urobilinogen

It is formed by reduction of bilirubin in the intestine. It reoxidizes to urobilin on exposure to air or when treated with mild oxidizing agents. It is a colourless crystalline solid, which shows no absorption bands in solution and no fluorescence on addition of zinc salts. Like urobilin it is precipitated by saturation with ammonium sulphate, but unlike urobilin it gives an immediate red colour with Ehrlich's aldehyde reagent. It is soluble in ether and light petroleum.

*Urobilin, stercobilin*, $C_{33}H_{46}O_6N_4$. Its structure is



Urobilin

It is a brown pigment formed by oxidation of the urobilinogen produced from bilirubin in the intestine by bacterial reduction. It can be separated from urine by saturation with ammonium sulphate and extraction with alcohol. In solution it shows a distinct absorption band (see p. 397) and gives a green fluorescence with zinc acetate in neutral solution.

## The Van den Bergh Reaction and the Serum Bilirubin

Methods of detecting and estimating bilirubin in serum are based on the formation of the purple compound azobilirubin, when bilirubin reacts with the diazo-reagent introduced by Van den Bergh.

### The Van den Bergh Reaction

This consists of two parts, the direct and indirect reactions. The

latter serves as a quantitative estimation of the serum bilirubin (Van den Bergh and Snapper, 1913 ; Van den Bergh and Muller, 1916).

**Reagents.** The diazo-reagent. Prepare freshly before use by adding 0·3 ml. of solution B to 10 ml. of solution A.

Solution A. Dissolve 1 gram of sulphanilic acid in 15 ml. of concentrated hydrochloric acid and make up to 1 litre with water.

Solution B. Dissolve 0·5 gram of sodium nitrite in water and make up to 100 ml. Prepare freshly at frequent intervals.

**Technique.** 1. **The Direct Reaction.** Pipette 0·5 ml. of serum into each of two small test tubes and add 0·5 ml. of water to one of them. This serves as a control with which the test can be compared. To the second tube add 0·5 ml. of the diazo-reagent, mix, and observe any colour change which occurs, noting the time after mixing of any such change.

Three different responses may be obtained :

(*a*) The *immediate direct reaction*, in which there is an immediate development of colour proceeding rapidly almost to a maximum. There is usually relatively little change in colour after three to four minutes have elapsed. The time taken to reach this maximum colour has been measured by repeating the test and observing the time required for the colours to become identical.

Van den Bergh differentiated between a prompt direct reaction which reached completion in thirty seconds, and a direct biphasic reaction in which colour development began immediately, but took longer to reach maximum intensity. This distinction has been abandoned by most workers.

(*b*) The *delayed direct reaction*, in which the colour only begins to appear from five to thirty minutes after mixing the serum and the diazo-reagent, and then develops slowly to a maximum. Differentiation from the immediate direct reaction is usually easy, but may be difficult when the amount of bilirubin present is small.

(*c*) *No direct reaction* may be observed.

2. **The Indirect Reaction. Determination of Serum Bilirubin.** The indirect reaction is essentially a method for the quantitative estimation of serum bilirubin and may be said to be positive when this is increased. Methods of estimating serum bilirubin can be divided into two groups according to whether or not the serum proteins are precipitated. Precipitation of protein was at first considered necessary if the whole of the serum bilirubin was to react with the diazo-reagent. However, several methods have been published recently in which this has been achieved without removal of the proteins. In Van den Bergh's original method, serum proteins were precipitated by addition of ethyl alcohol, and the diazo-reagent was added to the supernatant fluid obtained on centrifuging. Hence arose the term indirect reaction. The resulting colour, proportional to the amount of bilirubin present, was compared with suitable standards. Appreciable loss of bilirubin with the protein precipitate occurred. This was reduced by the modification introduced by Thannhauser and Anderson (1921 ; see also McNee and Keefer, 1925). King and

his co-workers have introduced further modifications (Haslewood and King, 1937 ; King and Coxon, 1950). In the last method King claims to have overcome this difficulty.

For the quantitative estimation of bilirubin, Van den Bergh first used as standard, solutions of pure bilirubin, then later a solution of cobaltous sulphate containing 2·16 grams of the anhydrous salt per 100 ml. of solution. This has the same colour as that given with the diazo-reagent by a solution of bilirubin in a final dilution of 1 in 4. Subsequently an aqueous solution of potassium permanganate and an ethereal solution of ferric thiocyanate have been used. Haslewood and King (1937) studied several of these methods and showed that a methyl red standard at *p*H 4·6 to 4·7 most closely resembles the colour of azobilirubin.

### Determination of Serum Bilirubin without Protein Precipitation

**I. Method of Malloy and Evelyn** (1937)

Serum is diluted with water, and methyl alcohol added in an amount insufficient to precipitate the proteins, yet sufficient to permit all the bilirubin to react with the diazo-reagent. Absolute methyl alcohol gives a clearer solution than 95 per cent. ethyl alcohol.

**Reagents.** 1. Absolute methyl alcohol.

2. Hydrochloric acid, 1·5 per cent. Dilute 15 ml. of the concentrated acid to 1 litre with water.

3. Diazo-reagent. Prepare freshly as above (p. 224).

4. Standard solution of bilirubin. Prepare a solution containing 10 mg. per 100 ml. chloroform (see below p. 227).

**Technique.** Take two test tubes and into each place 0·4 ml. of serum and 3·6 ml. of distilled water. To the unknown add 1 ml. of the diazo-reagent and to the blank 1 ml. of 1·5 per cent. hydrochloric acid. Finally, to each add 5 ml. of methyl alcohol. Stand for thirty minutes and read in the colorimeter using a yellow-green filter, or set at 540 millimicrons. Subtract the reading of the blank from that of the test.

A standard curve can be prepared as follows. Dilute the above standard solution 1 in 5 with methyl alcohol to obtain a solution containing 2 mg. bilirubin per 100 ml. Set up a series of tubes :

| Mg. bilirubin per 100 ml. serum | 0 | 2·5 | 5·0 | 10 | 15 | 20 | 25 | 30 |
|---|---|---|---|---|---|---|---|---|
| Ml. standard solution | 0 | 0·5 | 1·0 | 2·0 | 3·0 | 4·0 | 5·0 | 6·0 |
| Ml. methyl alcohol | 9·0 | 8·5 | 8·0 | 7·0 | 6·0 | 5·0 | 4·0 | 3·0 |
| Ml. diazo-reagent | Add 1 ml. to each tube. | | | | | | | |

and read as for the test.

The amount of *direct reacting bilirubin* can be determined in a similar way by substituting 5 ml. of water for the 5 ml. of methyl alcohol.

The disadvantage of this simple and otherwise very satisfactory

method which has been widely used is that, owing to the 1 in 25 dilution used, not much colour is developed when the value of the serum bilirubin is low. If more serum is used in an attempt to overcome this, some turbidity results from slight precipitation of protein. Hence methods have been devised in which an entirely aqueous solution is used. One such method is that of Powell who uses an aqueous solution of sodium benzoate and urea which gives almost immediate development of colour from the whole of the bilirubin present, at a 1 in 10 dilution, and so is more accurate with low concentrations of bilirubin than is the Evelyn method.

### II. Method of Powell (1944)

**Reagents.** 1. Diazo-reagent as before (p. 224).

2. Diazo-blank, 1·5 per cent. (v/v) hydrochloric acid.

3. Sodium benzoate-urea solution. Dissolve 10 grams of sodium benzoate in about 60 to 70 ml. of water, add 10 grams of urea, dissolve, and make up to 100 ml. Filter.

4. Standard solution of bilirubin, as before, containing 10 mg. per 100 ml. chloroform (p. 227).

5. A working standard containing 0·8 mg. per 100 ml. Prepare by diluting 4 ml. of stock solution to 50 ml. with 95 per cent. ethyl alcohol.

**Technique.** Total and direct reacting bilirubin can be estimated by setting up the following tubes :

1. *Total Bilirubin.* Test : 0·4 ml. serum, 0·2 ml. diazo-reagent, and 3·4 ml. sodium benzoate-urea solution.

Blank : 0·4 ml. serum, 0·2 ml. diazo-blank, and 3·4 ml. sodium benzoate-urea solution.

2. *Direct Bilirubin.* Test : 0·4 ml. serum, 0·2 ml. diazo-reagent, and 3·4 ml. water.

Blank : 0·4 ml. serum, 0·2 ml. diazo-blank, and 3·4 ml. of water.

Stand at room temperature for ten minutes and read in the colorimeter as for the Malloy and Evelyn method. Subtract the reading of the blank from that of the test. Powell diluted with distilled water in the case of high readings. Prepare a standard by mixing 5 ml. of the working standard given above, 1 ml. of diazo-reagent and 4 ml. of 95 per cent. alcohol. The colour this gives is read after thirty minutes and is equivalent to that produced by a serum containing 4 mg. bilirubin per 100 ml. Alternatively a standard curve can be prepared as described for the Malloy and Evelyn method, making due allowance for the difference in dilution, 1 in 10 in this case, as compared with the 1 in 25 of the former method.

Another method which avoids precipitation of proteins is that of Rappaport and Eichhorn (1943). They use a citric acid buffer with added caffeine and urea to keep the proteins in solution and render all the bilirubin reactive. By using a different buffer direct bilirubin can be estimated separately. The colour, however, is not so satisfactory as that given by the above methods.

### Determination of Serum Bilirubin with Protein Precipitation

**Method of King and Coxon** (1950)

Thannhauser and Anderson (1921), by developing the colour before precipitating the proteins, made use of the fact that azobilirubin is more soluble in alcohol than is bilirubin. Addition of ammonium sulphate was also found to help to separate the azobilirubin from the protein precipitate. King and Coxon have modified this method, and claim that they are now able to get results comparable with the Malloy and Evelyn method.

**Reagents.** 1. Diazo-reagent as before (p. 224).

2. Saturated ammonium sulphate solution.

3. Ethyl alcohol, 85 per cent. (v/v).

4. Methyl red standard. Dissolve 0·29 gram of methyl red in glacial acetic acid and make up to 100 ml. with the acetic acid. Pipette 1 ml. of this into a litre flask, add 5 ml. of acetic acid and a little water, and wash in 14·4 grams of sodium acetate ($CH_3COONa$, $3H_2O$) and dissolve. Make up to 1 litre with water. Take care to use pure methyl red. The colour of this standard matches that obtained from 0·1 mg. of bilirubin in a dilution of 1 in 25.

5. Phosphate buffer solution. Dissolve 3·6 grams of disodium hydrogen phosphate ($Na_2HPO_4$, $12H_2O$) in water and make up to 100 ml.

6. A standard solution of bilirubin. Ten mg. per 100 ml. of chloroform is suitable. As King points out, it may be necessary to reflux the mixture gently to dissolve the bilirubin.

**Technique.** Pipette 1 ml. of serum into a test tube and add 0·5 ml. of diazo-reagent. The nature of any direct reaction can be observed at this stage. Add 0·5 ml. of saturated ammonium sulphate solution and make to 10 ml. with 85 per cent. alcohol. Mix well and stopper. Lay almost horizontal for half an hour with the precipitate evenly distributed through the liquid. Filter and read in the photoelectric colorimeter with a green filter, or set at 520 millimierons. Use the methyl red standard.

**Calculation.** With a 1 in 10 dilution as above, the standard corresponds to 4 mg. per 100 ml. For the photoelectric colorimeter :

$$\text{Mg. bilirubin per 100 ml. plasma} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 4.$$

For values above 8 mg. per 100 ml. dilute the serum with the phosphate buffer. Whether or not this is necessary can be judged from the appearance of the serum.

The method can be checked against a bilirubin standard. King evaporated 20 ml. of the above bilirubin standard to 5 ml. and made up to 20 ml. with alcohol. The resulting solution, which contains 2·5 mg. per 100 ml., can be put up in the same way as the test.

*Quantitative determination of direct reacting bilirubin* can be carried out by mixing 1 ml. of serum with 0·5 ml. of diazo reagent, making up to 10 ml. with water and reading in the colorimeter.

**Note on Time used for reading Direct Reacting Bilirubin.** Various

methods have been used for studying the direct reacting bilirubin. Thus Ducei and Watson (1945) used a reading one minute after mixing the serum and diazo-reagent for the prompt direct reacting bilirubin. Gray and Whidborne (1947) read at thirty minutes for the total direct reacting bilirubin. The rate of colour development can also be studied. Malloy and Evelyn used 10, 30, 60, and 120 minutes. Such readings are only of limited value.

## Summary

The Malloy-Evelyn or Powell method is preferred. With the former, satisfactory results are obtained from 2 to 30 mg. per 100 ml., but below 2 mg. per cent. the colour is rather weak and cannot be read as accurately. In this lower range the Powell method gives a better colour and more reliable results. Of the methods which precipitate proteins, that of King and Coxon is the best. The methyl red standard of Haslewood and King can be used for these methods, corresponding in a 1 in 10 dilution, to 4 mg. bilirubin per 100 ml. of serum.

### VAN DEN BERGH REACTION AND SERUM BILIRUBIN. INTERPRETATION

A normal serum does not give a direct Van den Bergh reaction and contains not more than 1 mg. bilirubin per 100 ml. There is not complete agreement about the upper normal limit, which is sometimes stated to be a little higher.

In any consideration of the Van den Bergh reaction and serum bilirubin it is necessary to adopt a classification of the conditions in which jaundice may be found. Rolleston and McNee (1929) recognize three groups :

1. **Hæmolytic jaundice,** in which there is increased breakdown of hæmoglobin so that the normal liver cell is unable to excrete the increased amount of bilirubin formed. This occurs whenever there is excessive breakdown of red blood cells and is found in incompatible blood transfusion and icterus neonatorum ; following malaria and streptococcal septicæmia ; in the hæmolytic anæmias such as pernicious anæmia, familial and acquired acholuric jaundice and the rarer acute hæmolytic anæmias.

2. **Obstructive jaundice,** in which there is obstruction to the flow of bile in the extra-hepatic ducts, most often due to gall stones and carcinoma head of the pancreas.

3. **Hepatic or liver cell jaundice,** in which there is disease of the liver cells. Liver cell dysfunction is present in infective and toxic hepatitis, in acute and subacute hepatic necrosis, in yellow fever, Weil's disease, and in cirrhosis of the liver.

The correlation of the different types of Van den Bergh reaction with these three groups of jaundice is based on the hypothesis that bilirubin reacts differently with the diazo-reagent according to whether or not it has been excreted by the liver cells. Bilirubin formed from hæmoglobin—hæmobilirubin—has also been termed indirect bilirubin, since it does not give an immediate direct reaction, but only an indirect reaction (or occasionally a delayed direct reaction). Bilirubin which has been excreted by the liver cells—chole-

bilirubin—gives an immediate direct reaction, and has been termed direct bilirubin.

Accordingly, in hæmolytic jaundice there is an increase in indirect bilirubin, so that indirect reactions or direct delayed reactions are obtained. In obstructive jaundice, the increase is in direct reacting bilirubin so that an immediate direct reaction is found. In liver dysfunction it is assumed that both may be present. In jaundice due to acute liver disease, however, an immediate direct reaction is the rule, at least after the very early stages, presumably because the most important factor in causing the jaundice is an intrahepatic obstruction. In the low-grade jaundice which is present in some cases of cirrhosis of the liver, results are variable and an indirect reaction may be obtained. Attempts have been made, such as the direct biphasic reaction of Van den Bergh (p. 224), to recognize a type of reaction in liver disease due to the presence of both direct and indirect reacting bilirubin, which gives an immediate reaction developing only slowly to a maximum colour. These attempts have not been successful.

It will be seen that the same qualitative reaction is obtained, immediate direct, whether the obstruction is inside or outside the liver. This does not, therefore, differentiate between infective or toxic jaundice on the one hand, and obstructive jaundice due, for example, to carcinoma head of pancreas, on the other. Since this is the commonest problem in jaundice, the direct reaction is only of limited value, since the distinction which it makes does not usually give rise to difficulty in diagnosis. Many workers have almost ceased to use it and use the Van den Bergh reaction only as a quantitative estimation of serum bilirubin.

Another classification of jaundice, due to Rich (1930) and preferred by some workers, is into two groups, *retention jaundice*, in which there is impaired removal of bilirubin from the blood, and *regurgitation jaundice*, in which there is reabsorption into the blood of bilirubin which has been excreted by the liver cells. Retention jaundice is thus due to an increase in indirect reacting bilirubin and includes hæmolytic jaundice and those liver conditions characterized by impaired excretion of bilirubin, whilst regurgitation jaundice, in which there is an increase in direct reacting bilirubin, includes obstructive jaundice as described above and those liver conditions which have a considerable degree of intrahepatic obstruction. This classification serves to emphasize the failure of the direct reaction to distinguish whether a regurgitation jaundice is due to intra- or extra-hepatic obstruction.

There is no fully agreed explanation of the difference in behaviour of the serum bilirubin in these two groups of jaundice. A view commonly held is that it is due to the way in which indirect reacting bilirubin is attached to some of the plasma proteins. In support of this view, in addition to the behaviour after protein precipitation, is the observation that whereas direct bilirubin is excreted by the kidneys, this is not usually the case with indirect bilirubin (see p. 232). Several workers have found the direct bilirubin, but not the indirect,

to be dialysable, but there is not full agreement on this point. A further difference is that indirect bilirubin is soluble in chloroform whereas the direct reacting is not. For a discussion of this highly contentious subject, see With (1951), and Gray (1953).

*Determination of the Serum Bilirubin* gives a measure of the intensity of the jaundice. Higher levels are found as a rule in obstructive jaundice than in hæmolytic jaundice. In obstructive jaundice due to carcinoma head of pancreas, when the obstruction is complete the serum bilirubin may reach 20 to 30 mg. per 100 ml. Values as high as this may also be reached in acute infective hepatitis and in other forms of hepatic jaundice in which one supposes there is a considerable degree of intra-hepatic obstruction.

In pernicious anæmia the serum bilirubin rarely exceeds 4 mg. per cent. ; in acholuric jaundice figures up to about 10 mg. per 100 ml. are found. In the acute hæmolytic anæmias still higher values may be obtained.

The quantitative estimation of serum bilirubin is useful :

1. In sub-clinical jaundice, where the demonstration of small increases in serum bilirubin to between 1·0 and 3·0 mg. per 100 ml. is of diagnostic value.

2. In clinical jaundice, to follow the development and course of the jaundice. It gives a reliable and objective record of the degree of icterus and an earlier indication of any increase or decrease in the jaundice than does direct observation. In obstructive jaundice, particularly if the obstruction is due to some malignant growth, the serum bilirubin usually rises to a high level and remains almost constant about that figure. Only occasionally is an obstruction due to a tumour partial. A moderately raised serum bilirubin, of from 3 to 4 mg. per 100 ml., maintained for several weeks, has, however, been observed by the author in a case of cancer of the ampulla of Vater. If gall stones are lodged in the common bile duct the obstruction is rarely complete and the jaundice is variable. In infective hepatitis the serum bilirubin rises to a peak and then steadily returns to normal when recovery ensues. It is unusual for the serum bilirubin not to be falling by four or five weeks from the start of the jaundice.

**Ratio of Direct to Total Bilirubin.** Some workers have determined the ratio of direct to total bilirubin (Rappaport and Eichhorn, 1943 ; Gray and Whidborne, 1946, 1947). Gray and Whidborne used a *Direct Indirect Quotient* (DIQ) obtained by multiplying the ratio of the direct to the total bilirubin by 100. They find that if this is below 40 it is diagnostic of retention jaundice, if over 50 almost diagnostic of regurgitation jaundice. Between 40 and 50 it is more likely to be retention jaundice. Gray uses Evelyn's method for total bilirubin and compares it with the direct bilirubin similarly obtained at thirty minutes (see p. 225). This ratio, however, is only of limited value in the differential diagnosis of jaundice.

### Icteric Index (Meulengracht)

Icteric index measures the degree of jaundice by measuring the intensity of the yellow colour of the serum. Serum or plasma is

diluted with physiological saline until it matches in colour a 1 in 10,000 solution of potassium dichromate. The dilution factor is termed the Icteric Index.

**Reagents.** 1. Strong dichromate standard. Dissolve 0·1 gram of potassium dichromate, add 0·1 ml. of concentrated sulphuric acid and make to 100 ml. with water. Such a solution has an Icteric Index of 10.

2. One in 10,000 dichromate. Dilute the above 1 in 10.

3. Physiological saline. 0·9 per cent. sodium chloride.

**Technique.** Add physiological saline to 1 ml. of serum until the colour matches that of the 1 in 10,000 dichromate. The total volume in ml. then gives the Icteric Index. If the test is used frequently it may be convenient to prepare a series of tubes covering the range of Icteric Index from 1 to 10. These can easily be prepared by suitably diluting the strong dichromate standard. Alternatively the photo-electric colorimeter can be used. Dilute the serum until it roughly matches the 1 in 1,000 dichromate. Then read standard and unknown, using a blue-green filter, transmission 500 millimicrons. Then

$$\text{Icteric Index} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 10 \times \text{Dilution factor.}$$

Sometimes on diluting the serum some turbidity may result. Reinheld has used a phosphate buffer *p*H 7·0 for diluting, in order to prevent this. Addition of 1 drop of 10 per cent. sodium hydroxide is also usually effective. Lipæmia may also interfere with the comparison. It is important there should be no hæmolysis. If hæmolysis has occurred proteins should be removed. Mix 1 volume of serum with 2 volumes of acetone and filter or centrifuge. Compare as previously with standards. Make the necessary adjustment in the calculation.

**Note.** It will be seen that it is now no more trouble to determine the serum bilirubin than the icteric index and the results obtained are more reliable. It is recommended that serum bilirubin be used.

INTERPRETATION

The normal range is from 4 to 6. In latent jaundice the index is from 7 to 15. With an index over 15 clinically obvious jaundice should be present. In jaundice the icteric index parallels the serum bilirubin. Except for values under 1 mg. per 100 ml., it is roughly five times the serum bilirubin. The icteric index is not specific for bilirubin but is influenced by the carotenes present in the serum (see further, p. 414). These are plant pigments from which vitamin A is formed in the body. Ingestion of considerable amounts of carotenes in the diet can lead to a carotinæmia which increases the icteric index. If severe, a skin pigmentation which superficially resembles jaundice can result. The serum bilirubin, however, is normal in this condition so that it can be readily distinguished from jaundice in which both are increased together. Foods which are rich in carotenes include carrots, spinach, parsley, turnip tops, watercress, lettuce, tomatoes, of which carrots contain most.

## Tests for Bile Pigments in Urine

Most of the tests used depend on the oxidation of bilirubin to differently coloured compounds such as biliverdin, green, and bilicyanin, blue.

**Iodine Test.** This is one of the oldest. Dilute some tincture of iodine with 1 to 2 volumes of water and layer it carefully on to some of the urine in a test tube. A green ring at the junction of the two fluids indicates the presence of bilirubin. The test is not very sensitive and is often doubtful in cases where there is only a small amount present.

**Gmelin's Nitric Acid Test.** Layer carefully some urine on to a few ml. of concentrated nitric acid in a test tube. Green, blue, and violet rings are seen at the junction of the liquids if bilirubin is present.

The more delicate tests concentrate the bilirubin to some extent by bringing it down with a precipitate. Barium chloride solution is used to give such a precipitate with urine.

**Fouchet's Test.** Add a few ml. of 10 per cent. barium chloride solution to about 10 ml. of urine in a test tube. Filter, allowing to drain well. Spread out the filter paper on another dry paper and add a drop or two of Fouchet's reagent. To prepare this, dissolve 25 grams of trichloracetic acid in about 50 ml. of water, add 10 ml. of 10 per cent. ferric chloride, and make up to 100 ml. with water. A greenish-blue colour is obtained if bilirubin is present. This is a much more sensitive test than the Iodine and Nitric acid tests and is a good test to use for clinical purposes.

Other tests have used the Van den Bergh reagent, for example Godfried (1934) and Hunter (1930).

**Godfried Test.** Precipitate the bilirubin with barium chloride as in Fouchet's test. Add to the precipitate on the filter paper 1 drop of the Van den Bergh diazo-reagent, 4 drops of 95 per cent. alcohol and 1 drop of a phosphate solution (6 per cent. $Na_2HPO_4$, $12H_2O$). A red to pink colour is given by bilirubin. This is a most sensitive test.

**Hunter Diazo-test.** Make the urine acid if necessary, with acetic acid, and add 4 ml. of 10 per cent. barium chloride to 10 ml. of urine. Centrifuge. After washing the precipitate with water, add 0·5 ml. of the Van den Bergh diazo-reagent, 2 ml. of 95 per cent. alcohol and 0·3 ml. of the phosphate solution given above. A purple colour similar to that given by serum in the Van den Bergh reaction is given by bilirubin.

### INTERPRETATION

Bilirubin is found in the urine in obstructive jaundice and in most cases of liver cell jaundice. It is not present in the urine of most cases in hæmolytic jaundice. The kidneys do not appear to excrete indirect or blood bilirubin at the concentration in which it is usually present in blood in disease, but do excrete direct reacting or bile bilirubin when this is present above a small threshold amount, which may, however, vary. Thus Watson (1946) has reported bilirubin in the urine in the early stages of infective hepatitis before clinical jaundice had developed and when the serum level was 0·7 mg. per

100 ml., but did not find it when the jaundice was receding with blood levels as high as 3 mg. per cent. Pollock (1945) in a study of the biochemical findings in the early stages of infective hepatitis, used the Hunter diazo-test to detect bilirubin in the urine in the pre-icteric stage of this disease. The test was done on the early morning urine. He found the Hunter test positive in 86 per cent. of cases and as early as nine days before jaundice developed. It was negative in all normal cases and in all but 2 per cent. of non-hepatic diseases. The test can thus be used in epidemics of infective hepatitis for recognizing this condition in the prodromal stage, before clinical jaundice is manifest.

Gellis and Stokes (1945) used Franke's methylene blue test in the same way. Their results were similar to those of Pollock's. The Hunter test is more valuable, but the methylene blue test is simpler to perform.

**Methylene Blue Test.** Add 0·2 per cent. aqueous solution of methylene blue, drop by drop from a pipette which delivers 20 drops per ml., to 5 ml. of an early morning specimen of urine. Count the number of drops required to change the green colour of the mixture of urine and dye from green to blue. View by daylight. Negative tests require 1 to 4 drops. If five or more drops are needed the test is positive. Using the test for the early detection of epidemic hepatitis Gellis and Stokes obtained positive results in thirty-three cases from one to six days before the appearance of the jaundice. A thousand normal cases all gave negative results. The explanation of this test is not clear.

## Bile Pigments in Fæces

*Bilirubin* is not normally present in fæces since bacteria in the intestine reduce it to urobilinogen. Some may be found if there is very rapid passage of material along the intestine and it is sometimes found in the stools of very young infants. Bilirubin may be detected by Gmelin's test by adding a fæcal suspension to nitric acid as a ring test, or better by adding Fouchet's reagent (p. 232) to such a suspension. A green to blue colour is obtained if bilirubin is present.

*Biliverdin* is found in meconium, the material excreted during the first day or two of life.

## Urobilinogen

As stated, urobilinogen is formed in the intestine by bacterial action on bilirubin. The greater part is excreted in the fæces in which it exists as a mixture of urobilinogen and urobilin, the latter being formed by partial reoxidation of urobilinogen on exposure to air. These have also been known as stercobilinogen and stercobilin. Some urobilinogen is absorbed in the intestine but in normal persons this is mostly re-excreted by the liver. However, a little passes into the general circulation and is excreted in the urine. Urobilinogen accordingly can be found and estimated in both fæces and urine.

### Schlesinger's Qualitative Test for Urobilin in Urine and Fæces

*Urine.* To 5 to 10 ml. of urine in a test tube add 2 to 3 drops of tincture of iodine to convert the urobilinogen present into urobilin.

In another test tube shake about a gram of zinc acetate with a volume of absolute alcohol equal to the volume of urine taken. Mix the two well by repeated pourings backwards and forwards. Filter. Urobilin gives a greenish-yellow fluorescence due to the formation of zinc urobilin. Not more than a barely detectable amount of fluorescence is given by normal urines. Examine spectroscopically. Zinc urobilin shows a band in the green at the junction of the green and the blue (see Fig. 68, p. 397). Urines containing urobilin when acidified with hydrochloric acid have a spectrum with a rather broader band just in the blue (see Fig. 68), but this is not so easily seen as that of zinc urobilin in alcohol.

Before applying Schlesinger's test it is advisable to remove bile pigments. To do this add one-fifth volume of 10 per cent. calcium chloride solution to the urine. Filter and carry out the test on the filtrate.

Kirkpatrick (1953) has suggested a modified Schlesinger's test and claims this to be more sensitive. Add a few crystals of ammonium persulphate to 5 ml. of urine in a small separating funnel and shake to dissolve. Add 5 ml. of saturated alcoholic zinc acetate, mix, add 10 ml. of chloroform, and shake gently. Allow to separate and run the chloroform layer into a test tube. Clarify by mixing with a few drops of alcohol and examine in filtered ultra-violet light. A golden-yellow fluorescence shows the presence of urobilin. Small increases can be shown as follows. Divide the chloroform extract into two equal parts and add a crystal of trichloracetic acid to one. This destroys the zinc-urobilin complex so that yellow fluorescence in the untreated portion can be better seen when the two are compared together. A white or blue fluorescence constitutes a negative test.

*Fæces.* Extract a portion of fæces with a mixture of 20 parts alcohol and 1 part hydrochloric acid. Mix thoroughly and allow to stand for several hours or overnight. Examine the extract spectroscopically or apply Schlesinger's test. To do this neutralize with ammonia or sodium hydroxide and mix with an equal volume of alcoholic zinc acetate and filter.

### Wallace and Diamond's Qualitative Test for Urobilinogen in Urine
(Wallace and Diamond, 1925)

Add 1 ml. of Ehrlich's reagent, prepared by dissolving 2 grams of *p*-dimethylaminobenzaldehyde in 100 ml. of 20 per cent. hydrochloric acid, to 10 ml. of freshly voided urine. Allow to stand for three to five minutes for the colour to develop. Note the colour produced. Normal urines give not more than a faint red colour which is intensified by heating. Pollak uses the following classification :

| | |
|---|---|
| No red colour even on heating | Urobilinogen absent. |
| Faint pink in the cold | Urobilinogen present in normal amounts. |
| Distinctly red colour in the cold. | Increased amount of urobilinogen present. |

If the last of these is observed, assess roughly the extent of the increase by preparing a series of dilutions. Develop the colour as described above and dilute 1 ml. of the coloured urine to 10, 20, 30, 40, . . . 100 ml. of water and further if necessary, and find the dilution which last shows the faintest pink colour. No colour is given by normal urines diluted more than 1 in 20 in the cold.

Remove bilirubin by means of calcium chloride solution as described for Schlesinger's test.

**Determination of Urobilinogen in Urine. Watson's Method** (Watson, 1936)

Watson uses a weaker Ehrlich reagent and a solution of sodium acetate to bring out the maximum colour and to reduce the colour produced by indole and skatole. In this method any urobilin present is first reduced to urobilinogen by alkaline ferrous sulphate. The total urobilinogen is then extracted in acid solution by petroleum ether and determined colorimetrically by means of the colour given with Ehrlich's reagent, using Terwen's alkaline phenolphthalein standard.

**Reagents.** 1. Modified Ehrlich's reagent. Dissolve 0·7 gram of *p*-dimethylaminobenzaldehyde in 150 ml. of concentrated hydrochloric acid and add 100 ml. of water.

2. Ferrous sulphate, 20 per cent. solution. Powder 20 grams of $FeSO_4$, $7H_2O$ crystals, add 92 ml. of distilled water and dissolve. Or use 5 grams and 23 ml. of water for each determination.

3. Sodium hydroxide, 10 per cent. solution.

4. Petroleum ether, b. pt. 40° to 60° C.

5. Glacial acetic acid.

6. Saturated solution of sodium acetate in water.

7. Standard solution of alkaline phenolphthalein. Pipette 1 ml. of 0·05 per cent. phenolphthalein in alcohol and 5 ml. of saturated (20 per cent.) sodium carbonate solution into a 100 ml. measuring flask and make up to 100 ml. with water. The colour of a solution of urobilinogen containing 0·387 mg. treated with Ehrlich's reagent and diluted with water to 100 ml. was found by Terwen to give the same colour as this solution.

**Technique.** Collect a twenty-four-hour specimen of urine in a brown glass bottle containing about 5 grams of anhydrous sodium carbonate and 100 ml. of petroleum ether. Measure the volume of urine and place 50 ml. in a suitable flask. Add 25 ml. of the ferrous sulphate solution and then, with thorough mixing, add 25 ml. of 10 per cent. sodium hydroxide. Stand for at least half an hour. Filter. Acidify 50 ml. of the filtrate with about 5 ml. of glacial acetic acid and extract three times with about 30 ml. of petroleum ether shaking vigorously each time. Wash the combined extracts with water. Extract the urobilinogen by shaking thoroughly with 2 ml. of the Ehrlich's reagent. Add 4 ml. of saturated sodium acetate solution and again shake well. This brings out the colour. Any colour due to indole and skatole disappears as the hydrochloric acid is replaced by acetic acid on adding the sodium acetate, and excess aldehyde returns

to the petroleum ether. Repeat the extraction with Ehrlich's reagent and acetate to confirm the completeness of the first extraction. Continue until a colourless extract is obtained.

Compare the colour immediately with the standard, diluting if necessary to make the two solutions approximately similar in intensity. If the photoelectric colorimeter is used, use a yellow-green filter or transmission at 540 millimierons.

For high values use less filtrate. A rough preliminary test can be made as follows. To 2 ml. of filtrate add 2 ml. of Ehrlich's reagent and 4 ml. of acetate. Use 1 to 2 ml. of filtrate for the test if an intense red colour is produced, 10 ml. if a pale red colour results, 25 ml. with a pale pink, and 50 ml. if no colour is obtained.

**Calculation.** If the photoelectric colorimeter is used,

Mg. urobilinogen per twenty-four hours

$$= \frac{\text{Volume of urine and alkaline ferrous sulphate}}{\text{Volume of urine used}} \times \frac{\text{Volume of final extract}}{\text{Volume of filtrate used}} \times \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}387 \times \frac{\text{Ml. urine in the twenty-four hour specimen}}{100}.$$

If 50 ml. of urine is used, made up to 100 ml. with the alkaline ferrous sulphate, and 50 ml. of filtrate taken, then for one extraction with Ehrlich's reagent and sodium acetate the formula is

Mg. urobilinogen per twenty-four hours :

$$= \frac{100}{50} \times \frac{6}{50} \times \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}387 \times \frac{\text{Volume of twenty-four hour specimen}}{100}$$

$$= 0{\cdot}093 \times \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{\text{Volume of twenty-four hour specimen}}{100}.$$

Most normal urines contain between 0·5 and 1·0 mg. per diem. Watson gives the normal range as 0 to 4 mg., but few exceed 1 mg.

**Note.** Voegtlin (1948) has shown that low results may be obtained if the procedure after treating with alkaline ferrous sulphate is carried out in bright light. So it is recommended the determination be carried out in very diffuse light.

### Approximate Method of Schwarz, Sborow and Watson (1944)

**Reagents.** As for the above method.

**Technique.** Collect a single two-hour specimen of urine. The time recommended is from 2 p.m. to 4 p.m. The excretion of urobilinogen varies considerably and appears to be highest in the afternoon. Instruct the patient to empty the bladder at 2 p.m., then give a glass of water, and again have the bladder emptied two hours

later. Measure the volume of the specimen and cool to room temperature. Mix 2·5 ml. of urine with 2·5 ml. of Ehrlich's reagent, and add 5 ml. of saturated sodium acetate and again mix thoroughly. For a blank mix 2·5 ml. of urine and 5 ml. of sodium acetate, and then add 2·5 ml. of Ehrlich's reagent, slowly with constant shaking. Compare with the alkaline phenolphthalein standard in the photoelectric colorimeter as described for the previous method. The result is calculated as follows :

Ehrlich units per sample

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}387 \times 4 \times \frac{\text{Urine volume}}{100}.$$

Ninety per cent. of normal persons were found to be below 1 unit for the two-hour sample, the remainder being between 1 and 2 units.

This method gives higher values for the urobilinogen than the more accurate method given previously, but has the advantage that it is possible to make frequent examinations with little trouble.

**Determination of Urobilinogen in Fæces. Maclagan's Method** (Maclagan, 1946 (*a*) )

Watson used the same method for determining urobilinogen in fæces as for urine. He collected fæces for a four-day period, mixed well, took 10 grams, made a uniform suspension in 100 ml. of water, and added alkaline ferrous sulphate as described for urine. He thus determined the daily excretion. Maclagan simplified this proeedure and used a single specimen of fæces. This method is recommended.

**Reagents.** As for the urine method of Watson, p. 235.

**Technique.** Take a freshly formed stool and weigh out about 1·5 gram in a test tube. Emulsify with 9 ml. of water. Add 10 ml. of 20 per cent. ferrous sulphate and then 10 ml. of 10 per cent. sodium hydroxide, mixing well after each addition. Stand for one to three hours and then filter through an 11 cm. No. 1 Whatman filter paper. Pipette 2 ml. of the filtrate into a dry 250 ml. cylinder, add 2 ml. of Ehrlich's reagent, stand for ten minutes, add 6 ml. of saturated sodium acetate solution, and compare with the alkaline phenolphthalein standard, diluting if necessary. Measure the final volume in the cylinder. Use the photoelectric colorimeter as described for urine, if desired.

**Calculation.** Using the photoelectric colorimeter.

Mg. urobilinogen per 100 grams of fæces

$$= 3{\cdot}87 \times \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \text{Volume of the final coloured solution.}$$

This applies if 1·5 gram of fæces was taken. If a slightly different amount is used make the necessary correction.

Maclagan gives as the normal range 30 to 220 mg. per 100 grams of fæces. According to Watson 40 to 280 mg. of urobilinogen are excreted daily in the stools and 80 per cent. of values are between 100 and 250.

URINE AND FÆCAL UROBILINOGEN. INTERPRETATION

Since urobilinogen is formed in the intestine by the reduction of bilirubin, the amount of *fæcal urobilinogen* depends primarily on the amount of bilirubin entering the intestine. Fæcal urobilinogen is therefore increased in conditions in which there is increased breakdown of hæmoglobin to bilirubin, as in hæmolytic jaundice. In pernicious anæmia Maclagan found 220 to 400 mg. per 100 grams in five out of six cases, whilst in six cases of acholuric jaundice and chronic hæmolytic anæmia values of 400 to 1,400 mg. were obtained. The dark-coloured stools found in these conditions are due to the large amount of pigment present. The microcytic anæmias give low normal to low results because less hæmoglobin is being broken down.

Fæcal urobilinogen is reduced if there is any obstruction to the flow of bile into the intestine. The most complete degree of obstruction is found in obstructive jaundice due to tumour. Thus in malignant obstructive jaundice Watson found forty-nine out of fifty-two cases to have less than 5 mg. fæcal urobilinogen daily, with twenty-nine below 1 mg. The obstruction in gall stones is usually more intermittent and such low values are much less common. This may be useful in differentiating a non-malignant from a malignant obstruction.

In conditions such as infective hepatitis, in spite of the high degree of jaundice which may be present, values for fæcal urobilinogen are not nearly so low, as a rule, as in malignant obstruction. Watson found only two out of fifty-eight cases to have less than 5 mg. daily, and thirty-six to have over 20 mg. Maclagan found only 25 per cent. to have less than 30 mg. per 100 grams compared with 80 per cent. in obstructive jaundice. In cirrhosis of the liver the quantity of bilirubin entering the intestine tends to be reduced so that low normal to low values for fæcal urobilinogen are found.

Estimation of fæcal urobilinogen may thus be of value in revealing a high production of bilirubin in hæmolytic anæmia and in giving information as to the patency of the ducts.

The *urine urobilinogen* is derived from that part of the urobilinogen absorbed in the intestine which is not excreted by the liver. The amount present thus depends both on the amount of bilirubin entering the intestine and on the ability of the liver to excrete the urobilinogen coming to it from the intestine. Urine urobilinogen tends to be raised a little in hæmolytic jaundice since the liver is not able completely to excrete the increased quantity of urobilinogen absorbed from the intestine. Figures up to 10 mg. daily are found. In the microcytic anæmias, on the other hand, the amount is low.

In obstructive jaundice the complete, or almost complete, absence of bilirubin from the intestine is reflected in a very low urine urobilinogen. Watson found less than 0·3 mg. daily in forty-eight out of fifty-three cases of obstructive jaundice due to malignant tumours obstructing the bile ducts. As in the case of the fæcal urobilinogen, obstruction due to stones, being more intermittent, does not lead to such uniformly low values, and quite high amounts may be found

immediately following the passage of a stone which permits the entry of a large quantity of bile into the intestine.

In conditions such as infective hepatitis there is usually some bilirubin entering the intestine so that some urobilinogen is being formed. Although the amount of urobilinogen absorbed in the intestine may be less than in normal persons, the liver cell is less able to excrete it, so that more passes into the general circulation and is excreted in the urine. Consequently the very low values for urine urobilinogen seen in obstructive jaundice due to malignant obstruction of the ducts are much less commonly found in these conditions. Figures from normal to 50 mg. daily are quite frequently obtained. A small increase of amounts in the upper part of the normal range may be present even when the jaundice is at its height, with higher figures before and after the peak of the jaundice. As a result there is often quite a high urobilinogen excretion whilst the bilirubin is falling, in fact the urobilinogen rises when the bilirubin begins to fall. In infective hepatitis and similar conditions positive tests for both bilirubin and urobilinogen in the urine are obtained.

Similarly in cirrhosis of the liver, in spite of the reduced amount of urobilinogen formed in the intestine, because there is liver cell damage there may be appreciable increase in the urine urobilinogen which may be of diagnostic value for in these cases bilirubin may not be present in the urine.

### Tests for Bile Salts in Urine

**Hay's Test.** This test is based on the fact that bile salts when present in the urine lower the surface tension.

Place some fresh clear urine in a small beaker at room temperature. Sprinkle a little dry, finely powdered flowers of sulphur on to the surface. The sulphur particles sink in the presence of bile salts but remain floating if they are absent. Urines preserved with thymol may give a false positive, and so should not be used.

**Pettenkofer's Test.** Add 5 drops of 5 per cent. sucrose to about 5 ml. of urine in a test tube, slope the tube and carefully pour 2 to 3 ml. of concentrated sulphuric acid down the inside. A red ring is obtained if bile salts are present. Shake the tube gently whilst holding it under running water. The red colour spreads throughout the liquid. It is essential that the liquid be kept cool, under 70° C. This test is less sensitive than the Hay's test. Protein and urine pigments may give interfering colours.

INTERPRETATION

Bile salts appear to be formed in the liver. Excreted in the bile they are absorbed by the intestine and passed back to the liver through the portal circulation. Their main function is in connection with fat digestion due to their power to reduce surface tension and so act as emulsifying agents. Bile salts are present together with bile pigments in the urine in obstructive jaundice. In some cases with liver damage there is a smaller amount of bile salts in proportion to pigment and there may sometimes be absence of bile salts though pigments are present.

### Determination of Bile Salts in Blood

This is complicated. Methods available are those of Gregory and Pascoe (1929); and Lichtmann (1934). Josephson (1935) gives a method for estimating cholic acid.

## TESTS BASED ON CARBOHYDRATE METABOLISM

Several tests of hepatic function based on the role played by the liver in carbohydrate metabolism have been used. When the liver cell is damaged a diminished rate of glycogen formation results so that more of the sugar absorbed from the alimentary tract into the portal circulation passes into the peripheral circulation. To test this function of the liver, monosaccharides have been mainly employed. The glucose tolerance test is not, however, of much value in liver disease for although glucose tolerance is sometimes diminished it is often difficult to separate the part played by the liver from other factors influencing glucose metabolism. Soskin has suggested that the intravenous glucose tolerance test has some value in this connection (see p. 52). It is easier to interpret tests in which either galactose or fructose are taken and these have been most frequently used.

### Galactose Tolerance Tests

In its original form (Bauer) 40 grams of galactose was given by mouth, and the amount of galactose excreted in the urine during the following five hours was determined. Less than 3 grams of galactose should be passed in that time. More recently two other methods of carrying out the galactose tolerance test have been suggested, one by Maclagan and the other by King. Both use determination of blood galactose following the giving of galactose, but whereas Maclagan gives the galactose by mouth, King uses intravenous injection. The tests are more reliable than the older urinary test.

### Oral Galactose Tolerance Test. Maclagan (1940)

The test is performed in the morning after a night's fast. Dissolve 40 grams of galactose in a cupful of water with the aid of a little heat if necessary. Take a 0·2 ml. specimen of blood, fasting, for use as a blank. Put this into one of the yeast tubes prepared as described below. Give the galactose and take further 0·2 ml. blood samples at half-hourly intervals for two hours afterwards. Place these also into yeast tubes, and determine the amount of galactose present.

### Determination of Blood Galactose

This is comparatively easy since the glucose can be removed by fermentation with yeast, after which the galactose can be determined by any of the standard blood sugar methods. Maclagan uses a modified Shaffer Hartmann method which is described.

**Reagents.** 1. Isotonic sodium sulphate solution. Dissolve 3 grams of $Na_2SO_4$. $10H_2O$ in water and make up to 100 ml.

2. Sodium tungstate. 10 per cent. solution of $Na_2WO_4$. $2H_2O$.

3. Copper sulphate, 7 per cent. solution of $CuSO_4$. $5H_2O$.

4. Alkaline copper solution. Dissolve 25 grams of anhydrous sodium carbonate, 20 grams of sodium bicarbonate, and 25 grams of Rochelle salt in about 600 ml. of water. Dissolve 7·5 grams of copper sulphate ($CuSO_4$. $5H_2O$) separately in about 100 ml. of water. Mix in the copper sulphate slowly, with constant stirring, through a funnel the tip of which rests on the bottom of the beaker. Add 5 grams of potassium iodide and 0·175 gram of potassium iodate ($KIO_3$). Bring these into solution and make up to 1 litre.

5. Normal sulphuric acid.

6. N/500 sodium thiosulphate. Prepare freshly before use by diluting 2 ml. of stock N/10 to 100 ml. with water.

7. One per cent. starch solution in saturated sodium chloride solution.

8. Standard galactose solution containing 100 mg. per 100 ml.

9. Yeast tubes. Prepare as follows: place 1 ml. of a 1 in 3 suspension of fresh baker's yeast in water into a centrifuge tube, add 10 ml. of distilled water, mix thoroughly with a glass rod and centrifuge. Decant the supernatant fluid and repeat the washing twice with further 10 ml. of water. Drain the tube and wipe the inside with filter paper. Then add 2·2 ml. of the isotonic sodium sulphate solution.

**Technique.** Add 0·2 ml. of blood to the yeast tubes, mix thoroughly and allow to stand for at least thirty minutes at room temperature. Six of these tubes should be set up, one containing the fasting blood used as a blank to show that the yeast is active, the four specimens of the test, and a further tube into which is placed 0·2 ml. of galactose-free blood and 0·2 ml. of the standard galactose solution. This last tube should contain 2 ml. of isotonic sodium sulphate so as to give the same total volume. This tube affords a control that the yeast does not act on galactose and checks a point on the calibration curve.

After standing to allow fermentation of the glucose, add 0·3 ml. of 10 per cent. sodium tungstate, mix thoroughly, add 0·3 ml. of 7 per cent. copper sulphate and mix again. Centrifuge for a few minutes. Pipette 2 ml. of each of the supernatant fluids into a series of test tubes and add 2 ml. of the alkaline copper solution. Mix by shaking and plug lightly with cotton wool. Place the tubes in briskly boiling water for ten minutes, cool in running water for two minutes, immediately add 2 ml. of N sulphuric acid, shake, and titrate with the N/500 thiosulphate using a 5 ml. micro-burette, adding 1 drop of the starch solution as indicator. The blank requires about 4·9 ml. of the thiosulphate.

**Calculation.** Titration of blank minus titration of unknown gives a measure of the amount of galactose present. Since the relationship between the thiosulphate titre and the galactose content is not quite linear, particularly for low values, a calibration curve should be prepared. To do this, set up a series of yeast tubes containing 0·2 ml. galactose-free blood in 2 ml. of isotonic sodium sulphate and 0·2 ml. of galactose solutions of known strength, for example, 0, 20, 60, 100,

200 mg. per 100 ml., preferably in duplicate. The blood galactose values in the test can then be read directly from this calibration curve.

INTERPRETATION

Maclagan uses a Galactose Index obtained by adding the four blood galactose values. He gives 160 as the upper limit of normal. In medical students he obtained a range of 0 to 110, but in hospital patients not known to be suffering from liver disease, the range was 0 to 160. Much increased values may be found in liver disease. In infective and toxic hepatitis values up to about 500 are seen, decreasing only slowly as the clinical condition of the patient improves. Even when the jaundice has disappeared the galactose index may still be raised. This is a characteristic of all liver function tests in acute liver damage. In cirrhosis of the liver increased values may be obtained up to 300 to 400 or more, depending on the severity of the disease. Maclagan and Rundle (1940) also found consistently increased figures in hyperthyroidism.

**Intravenous Galactose Tolerance Test** (King and Aitken, 1940)

King suggested an intravenous form of the test in which blood galactose is estimated five minutes, half, one, one and a half, and two hours after the intravenous injection of galactose equivalent to 0·5 gram per kilogram of body weight, given as a sterile 50 per cent. solution. According to this a normal person should have a curve beginning on the average at about 200 mg. galactose per 100 ml., falling steeply during the first hour and reaching a figure between 0 and 10 mg. per cent. by the end of two hours. In most cases of obstructive jaundice, similar results are obtained, there being little or no galactose in the blood at the end of the two hours. In conditions in which there is liver cell damage, the fall in blood galactose takes place more slowly.

Though this intravenous test avoids errors due to impaired absorption of galactose from the intestine, in the great majority of cases the Maclagan test gives similar information and is to be preferred on account of its greater simplicity. It is also more useful than the lævulose tolerance test. Lævulose is much less rapidly absorbed from the intestine than galactose, so that in health there is little or no rise in total blood sugar after lævulose is ingested.

**Lævulose Tolerance Test**

Fifty grams of lævulose is given to the fasting patient, prepared as for the glucose tolerance test. The blood sugar is estimated before, and at half-hourly intervals for two and a half hours after taking the lævulose. The usual methods for estimating blood sugar measure both the glucose and lævulose present. The normal response shows little or no rise in the blood sugar level (see column 1, Table XIV). The highest blood sugar reached during the test should not exceed the fasting level by more than 30 mg. per 100 ml. The rise in blood sugar is greater than this when there is liver cell damage but the increases obtained are never great. In column II of the table there is shown an abnormal response from a case of infective hepatitis.

TABLE XIV

*Lævulose Tolerance Tests*

| | I | II |
|---|---|---|
| Fasting blood sugar . . . . . | 95 | 85 |
| Blood sugar ½ hour after 50 grams lævulose . | 100 | 130 |
| Blood sugar 1 hour after 50 grams lævulose . | 105 | 145 |
| Blood sugar 1½ hours after 50 grams lævulose. | 100 | 150 |
| Blood sugar 2 hours after 50 grams lævulose . | 95 | 135 |
| Blood sugar 2½ hours after 50 grams lævulose. | 90 | 120 |

Blood lævulose cannot be estimated in the same way as blood galactose, since yeast ferments both lævulose and glucose. Special colorimetric methods are used (Stewart, Searborough and Davidson, 1937, 1938 ; Davidson *et al.* 1946). Herbert and Davison (1938) have used this technique in the lævulose tolerance test, measuring the blood lævulose alone, instead of the total blood sugar.

## TESTS DEPENDING ON ABNORMALITIES IN PLASMA PROTEINS

### Determination of the Plasma Proteins

This yields most useful information in chronic liver disease. For methods of estimation, see pp. 164–72. The liver is the site of albumin and fibrinogen synthesis. In advanced liver disease the albumin is decreased and the globulin often increased, so that the albumin-globulin ratio may be reversed. The *albumin* may fall below 2·5 per cent. and is a contributory factor in causing the œdema present in such cases. The *globulin*, on the other hand, may be increased to over 4 and 5 per cent., a level which is said to indicate a bad prognosis (Higgins *et al.*, 1944). Fractionation of globulin reveals that the increase is mainly in the $\gamma$-globulin. In acute hepatitis, quantitative estimation of albumin and globulin gives normal results in the early stages, though there is evidence that qualitative changes may be present (see p. 245). In some cases values for albumin fall within the lower normal range. Normal figures are also the rule in obstructive jaundice, at least so long as there is no complicating liver cell damage.

*Fibrinogen*, formed in the liver, is normal (200 to 400 mg. per 100 ml.) unless considerable liver damage has occurred. Then, values below 100 mg. per 100 ml. have been reported. These are found in severe acute liver insufficiency such as may occur in acute yellow atrophy, in poisoning from carbon tetrachloride, and in the advanced stages of liver cirrhosis.

### Flocculation Tests

Flocculation tests depend on an alteration in the type of protein present in the plasma. This may be either qualitative or quantitative

and most frequently involves one or more of the globulin fractions. Since all these tests depend on rather similar changes in the plasma proteins, it is not usual to employ more than one or two of them.

One of the earliest was the *Takata-Ara* reaction. First used on cerebrospinal fluid the test was applied to blood serum in pneumonia and then later by Jezler to cases with liver disease. In carrying out the test, place 1 ml. of 0·9 per cent. sodium chloride solution into eight small tubes. Add 1 ml. of serum to the first, mix, transfer 1 ml. to the second tube, mix, and proceed similarly along the row of tubes so that a series of dilutions of serum from $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ . . . $\frac{1}{256}$ is obtained. To each tube add 0·25 ml. 10 per cent. sodium carbonate solution and 0·3 ml. of a solution made by mixing equal volumes of 0·5 per cent. mercuric chloride and 0·025 per cent. fuchsin in water. Normal serum only precipitates from tube 4 onwards. A precipitate in tube 3 or earlier constitutes a positive reaction.

Although the test is positive in cirrhosis of the liver, it is by no means specific for liver dysfunction, and is not nearly so satisfactory as the tests to be described.

### The Thymol Turbidity Test (Maclagan 1944 (*b*))

The degree of turbidity produced when serum is mixed with a buffered solution of thymol is measured.

**Thymol Buffer.** Add 500 ml. of distilled water to 1·38 grams of barbitone, 1·03 grams of sodium barbitone and about 3 grams of thymol. Heat the mixture just to boiling point, shake well, and cool thoroughly. It will be turbid. Seed with a small amount of powdered thymol crystals, shake, and allow to stand overnight at a temperature of 20° to 25° C. Shake well and filter. Store in a slightly warmish place in cold weather, at about or near to 20° C., since there is a significant reduction in the solubility of thymol below 20° C.

**Technique.** Pipette 0·10 ml. of serum into a dry test tube and add 6 ml. of the thymol buffer. Allow to stand for half an hour. Compare with the set of protein standards containing 10, 20, 30, 40 . . . 100 mg. per 100 ml. described for the estimation of cerebrospinal fluid protein by the method of Kingsbury (see p. 462).

Maclagan expresses the result in units so that a turbidity equivalent to that of the 10 mg. per 100 ml. standard is 1 unit ; that equivalent to the 20 mg. per cent. standard 2 units, and so on up to 10 units, when the turbidity matches that of the 100 mg. per 100 ml. standard. If the turbidity exceeds that of the 100 mg. per cent. standard, dilute with the thymol buffer so that the turbidity reads under 10 units and make the necessary adjustment in the calculation.

As described under cerebrospinal fluid protein, a standard curve can be prepared for the photoelectric colorimeter, so that the turbidity can be read on that instrument instead of comparing with the standards. Use a red filter or transmission at 650 millimicrons. Maclagan, however, still prefers visual comparison and considers it to be more reliable.

**Notes.** 1. The *p*H of the above thymol buffer is 7·8. It has been suggested that a buffer of *p*H 7·55 gives greater sensitivity. For this

the buffer is made up using 1·519 grams of barbitone and 0·929 gram of sodium barbitone. The sensitivity appears to be increased but possibly at the cost of some degree of specificity. Maclagan (1947) sees no need for such a readjustment and continues to use the original buffer, *p*H 7·8, and this is recommended since uniformity of technique is desirable.

2. Shank and Hoagland (1946) suggested a set of suspensions of barium sulphate as standards (see also Ley *et al.*, 1946). These do not compare accurately with the Maclagan standards but give readings approximately twice as great. Shank and Hoagland used a 0·0962 N solution of barium chloride (1 per cent. $BaCl_2$). When 3 ml. of this is made up to 100 ml. with 0·2 N sulphuric acid, the resulting turbidity corresponds to 10 Maclagan units, not 20 as stated by these authors. As Ducci (1947, a) points out, for a turbidity equal to 20 units 0·0962 M barium chloride solution must be used.

INTERPRETATION

The usually accepted normal range is 0 to 4 units, but the great majority of normal persons are between 1 and 2 units. For hospital patients, however, the wider range is used. The following figures are from Maclagan's original paper.

| | No. of cases | Per cent. positive | Thymol units | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0–4 | 5–7 | 8–10 | 11–15 | 16—20 | Over 20 |
| Infective hepatitis . . | 134 | 91 | 12 | 22 | 51 | 30 | 14 | 5 |
| Cirrhosis . . . . | 13 | 100 | 0 | 2 | 4 | 3 | 1 | 3 |
| Arsenical jaundice . . | 32 | 47 | 17 | 11 | 2 | 1 | 1 | 0 |
| Weil's disease . . . | 23 | 52 | 11 | 9 | 3 | 0 | 0 | 0 |
| Amyloidosis . . . | 7 | 14 | 6 | 1 | 0 | 0 | 0 | 0 |
| Obstructive jaundice . . | 39 | 8 | 36 | 3 | 0 | 0 | 0 | 0 |

(From *British Journal of Experimental Pathology*. H. K. Lewis & Co. Ltd.)

In infective hepatitis the thymol turbidity is usually highest soon after the onset of the jaundice, but frequently remains raised for several weeks and occasionally even for months after the serum bilirubin has returned to within normal limits.

Maclagan and Bunn (1947) investigated the factors which govern the development of turbidity in liver disease. They found the γ-globulin to be mainly responsible. The test also depends on the phospholipids present. Moreover, qualitative changes of the proteins appear to be important, particularly of the albumin, for whereas normal serum albumin was found to inhibit the effect of the γ-globulin, the albumin from a case of infective hepatitis did not.

**Thymol Flocculation.** It was observed that when the thymol turbidity test was allowed to stand overnight, flocculation sometimes occurred. This was made the basis of a thymol flocculation test by Neefe (1946 ; see also Maclagan, 1947). After the turbidity has been measured, leave the tube to stand in the dark overnight (that is for eighteen hours) and read the degree of flocculation. This may be graded : Negative (no flocculation) : 1+; 2+; 3+; 4+.

The flocculation test proves to be positive in some cases in which the turbidity test is found to be negative. It is claimed that the sensitivity of the thymol turbidity test is increased by this additional observation (see also Shay *et al.*, 1947).

### The Serum Colloidal Gold Test

A serum colloidal gold test was introduced by Gray (1940) in which serum diluted 1 in 350 was put up in a colloidal test in the same way as cerebrospinal fluid (see p. 466). The test was further studied by Maclagan (1944 (*a*) ; 1946 (*b*) ), who developed a one tube test in which a barbitone buffer *p*H 7·7 and gold sol are added to serum. The degree of precipitation which results after standing overnight is assessed.

**Reagents.** 1. Barbitone buffer, *p*H 7·7. Add 100 ml. of distilled water to 0·55 gram of barbitone, 0·31 gram of sodium barbitone and 0·2 gram of phenol. Dissolve by heating gently. This solution keeps for at least two to three months.

2. Gold sol prepared as for the Lange gold curve on cerebrospinal fluid (p. 466). The *p*H of the final product should be about 5·9. Cover the flask with a beaker. Keep in the dark. Do not pipette directly from the flask and do not return unused portions to the stock solution.

**Technique.** Pipette 0·05 ml. of serum in an accurately graduated 0·2 ml. pipette with a waxed tip into a clean pyrex tube (6 × $\frac{5}{8}$), add 0·5 ml. of the barbitone buffer and then 2·5 ml. of the gold sol. Shake, stand overnight, and read the amount of precipitation. Maclagan records complete precipitation as 5+, slight precipitation, just visible to the naked eye as 1+, with intermediate grades of precipitation as 2+, 3+, and 4+, according to the colour of the supernatant fluid. Normal serum gives no precipitation at all, which is recorded as 0.

INTERPRETATION

Results obtained in liver disease roughly parallel those given by the thymol turbidity test. Thus Maclagan (1944 (*a*) ), obtained 92 per cent. positive tests in infective hepatitis, 47 per cent. in post-arsphenamine jaundice, 87 per cent. in hepatic cirrhosis, but only 8 per cent. in obstructive jaundice.

The reaction appears to depend on an increase in the $\gamma$-globulin and on a decrease in serum albumin. Maclagan (1948) states that in this test, the $\alpha$- and $\beta$-globulins as well as the albumin, counteract the effect of the $\gamma$-globulin.

### Colloidal Red Test (Ducci, 1947 (*b*) )

This test gives results similar to those obtained with the colloidal gold test. It has the advantage that the colloidal red solution may be easier to prepare.

**Reagents.** 1. Saturated alcoholic solution of scarlet red (Scharlach R, Sudan IV). Shake some dye in a bottle with absolute alcohol. Store in incubator for twenty-four hours before using.

2. Buffer, *p*H 7·53. Dissolve 0·325 gram of sodium barbitone, 0·639 gram of diethylbarbiturie acid and 0·2 gram of phenol in 90 ml. of water with the aid of heat, eool, and make up to 100 ml. Cheek the *p*H and adjust if neeessary.

3. Scarlet red reagent. Warm 10 ml. of stock scarlet red solution and 50 ml. of water separately to 55° C. Add the water quiekly and boil, first in a water bath, then over a gauze, until the volume is reduced to 20 ml. Make up to 800 ml. with distilled water. Keep in the refrigerator.

**Technique.** Mix 0·05 ml. of serum, 0·5 ml. of buffer and add 2·5 ml. of the scarlet red reagent. Read as follows after standing for twenty-four hours :

0 No change.
1 Faint precipitate.
4 Slightly eoloured supernatant fluid, with heavy precipitate.
5 Clear supernatant fluid, with heavy precipitate.
2 and 3 are intermediate, mainly judged by the eolour of the supernatant fluid.

**The Cephalin Cholesterol Flocculation Test** (Hanger, 1939 ; Pohle and Stewart, 1941 ; Hanger and Patek, 1941)

This test was introduced by Hanger who studied the ability of the serum from eases of hepatie disease to floeculate a eolloidal cephalin eholesterol suspension.

**Preparation of the Cephalin Cholesterol Suspension.** A eephalin eholesterol preparation is available (Difeo, British agents, Baird and Tatlock) from which the suspension as used in the test ean easily be prepared. Otherwise it ean be made as follows.

Prepare the eephalin from sheep's brain. Dehydrate fresh maeerated brain tissue by three extractions with aeetone. Powder the dry material obtained and extract three times with peroxide-free ether. Coneentrate the ether extracts *in vacuo* and precipitate the erude eephalin by addition of 4 volumes of absolute aleohol. Dissolve the preeipitate obtained, in the smallest possible amount of ether, chill to precipitate eerebroside impurities, and eentrifuge. Again precipitate the remaining ether solution with 4 volumes of absolute aleohol, chill, filter the precipitate, wash with aleohol and aeetone, and dry. From this, prepare the stock eephalin eholesterol solution by dissolving 100 mg. of the eephalin and 300 mg. of eholesterol in 8 ml. of ether.

**Preparation of the Cephalin Cholesterol Emulsion for Use.** Add slowly, with stirring, 1 ml. of the stock ether solution as prepared above, to 35 ml. of freshly distilled water warmed to 65° to 70° C. Heat slowly to boiling and allow to simmer until the volume is reduced to 30 ml. A milky translueent emulsion is prodneed and the ether completely driven off. Cool to room temperature. The emulsion is then ready for use.

**Technique of the Test.** Dilute 0·2 ml. of the serum, which is to be tested, with 4 ml. of normal saline (0·9 per eent.) and add 1 ml. of the emulsion. Shake thoroughly, stopper the tube with cotton wool,

stand upright in a rack and leave undisturbed at room temperature. Read the amount of floeculation after twenty-four and forty-eight hours.

Normal sera show no change in the state of the emulsion, the test being described as negative. With positive tests, grading is based on the amount of floeculation which has taken place. 4+ indicates complete precipitation, leaving a completely clear supernatant fluid. 1+, 2+, 3+ show intermediate degrees of flocculation. Each batch of emulsion should be tested with known negative and known 4+ sera. These control sera can be kept for at least a month frozen solid in the refrigerator.

Serum used for the test should be fresh and put up within five to six hours of taking the blood. Glassware should be cleaned thoroughly with acid, steamed and washed with distilled water.

INTERPRETATION

The test gives results which roughly parallel those of the thymol turbidity test. It is positive in the early stages of infective hepatitis before the jaundice develops. Comparison with the thymol turbidity test shows that, in general, the cephalin cholesterol test becomes positive several days earlier in this condition than does the thymol turbidity test, whilst the latter remains positive for a longer period. The test has been used in the diagnosis of the pre-icteric stage of infective hepatitis during epidemics. When a positive test persists after apparent recovery from an attack of hepatitis this indicates a poor prognosis.

The test resembles the thymol turbidity test in showing a much reduced percentage of positive results in arsenical jaundice. Negative tests are found in uncomplicated obstructive jaundice. Whilst normal albumin has an inhibiting effect, $\gamma$-globulin and probably $\alpha$ and $\beta$ globulins have a precipitating action.

Thus, as with the other floeculation tests, this is best regarded as an empirical test to detect active liver damage.

Compared with the thymol turbidity test the cephalin cholesterol test is not so easy to read and successive batches of emulsion are not as reproducible as the thymol buffer. Its main advantage is in the earlier stages of infective hepatitis.

### The Zinc Sulphate Test (Kunkel, 1947)

Whilst the floeculation and turbidity tests so far described depend for their effects on alterations in $\gamma$-globulin, other factors are involved. Kunkel has described a zinc sulphate test which he claims gives an index of the amount of $\gamma$-globulin present. Serum is mixed with a buffered zinc sulphate solution and the turbidity produced is read in the same way as in the thymol turbidity test.

**Reagent.** Buffered zinc sulphate solution. Dissolve 24 mg. of zinc sulphate, $ZnSO_4$, $7H_2O$, 280 mg. of barbitone and 210 mg. of sodium barbitone in distilled water and make up to 1 litre. The *p*H is 7·5.

**Technique.** Pipette 1 volume of serum (0·1 ml.) and add it to

60 volumes of the buffered zinc sulphate (6 ml.). Stand for thirty minutes, shake, and read the degree of turbidity in the same way as described for the thymol turbidity. Kunkel used the barium sulphate standards of Shank and Hoagland (1946). Three ml. of a solution containing 1·15 grams of $BaCl_2$, $2H_2O$ per 100 ml. are made up to 100 ml. in a volumetric flask with 0·2 N sulphuric acid. The turbidity of this solution is said to be equivalent to 20 units.

In the absence of standards a rapid estimate of the globulin increase can be made by visual observation of the turbidity. The time required for flocculation to take place gives a measure of the $\gamma$-globulin level. Some sera with high globulin content flocculate in a few minutes while normal sera do not flocculate for at least twelve hours. Patients with slight $\gamma$-globulin increase usually flocculate within four hours.

INTERPRETATION

The normal range was found to be between 2 and 8 units with results up to 80 units in disease. All cases of cirrhosis of the liver gave positive results. Kunkel observed a delayed rise in $\gamma$-globulin in infective hepatitis with subsequent prolonged high levels and suggested that this might reflect the production of antibodies rather than be a disturbance in liver function. Serial determinations of thymol turbidity ran parallel with results from this test during convalescence from infective hepatitis but marked differences occurred in cirrhosis. Kunkel reported five cases which developed chronic hepatitis following infective hepatitis and stated that all showed raised $\gamma$-globulin. He claimed that the zinc sulphate test is the most sensitive for detecting a residual hepatitis.

Since the test depends on increase in $\gamma$-globulin it is not specific for liver disease and gives positive results in other conditions such as multiple myeloma in which globulin is altered. Kunkel used the test to follow changes in $\gamma$-globulin in scarlet fever and rheumatic fever.

## Weltmann Coagulation Test

Weltmann (1930) introduced a sero-coagulation test. A constant amount of serum is added to different concentrations of calcium chloride and heated in a water bath. With abnormal fluids coagulation may cease to occur at higher or lower concentrations than is the case with normal sera.

**Reagent.** Ten per cent. calcium chloride solution. Dissolve 10 grams of $CaCl_2$. $6H_2O$ in water and make up to 100 ml.

**Technique.** Place ten small tubes in a rack. Dilute the stock calcium chloride solution 1 in 100 to obtain a 0·1 per cent. solution. Set up tubes as follows :

| Tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Per cent. $CaCl_2$. $6H_2O$ | 0·1 | 0·09 | 0·08 | 0·07 | 0·06 | 0·05 | 0·04 | 0·03 | 0·02 | 0·01 |
| Ml. 0·1 per cent. $CaCl_2$. $6H_2O$ | 5·0 | 4·5 | 4·0 | 3·5 | 3·0 | 2·5 | 2·0 | 1·5 | 1·0 | 0·5 |
| Ml. distilled water | 0 | 0·5 | 1·0 | 1·5 | 2·0 | 2·5 | 3·0 | 3·5 | 4·0 | 4·5 |
| Serum | Add 0·1 ml. to each tube. | | | | | | | | | |

Shake well to mix, and place in a bath of boiling water for fifteen minutes. The tubes may remain clear, show opalescence, show turbidity, or flocculate.

Weltmann refers to the number of tubes in which there is coagulation as the coagulation band. Normal sera show flocculation in the first six tubes, with possible slight change in the seventh. This is described as a coagulation band of 6 or $6\frac{1}{2}$. In case of doubt or slight coagulation, $\frac{1}{2}$ is added to the number of the last tube showing undoubted coagulation. Weltmann described the reaction as being moved to the right if the coagulation band is 7 or greater, and to the left if below 6, thus giving a long or short coagulation band, respectively.

INTERPRETATION

This is not a specific diagnostic test but is claimed to be altered in parenchymal liver disease. A longer coagulation band occurs in acute hepatic jaundice and in liver cirrhosis, the test being normal in obstructive jaundice. It has been used in lung conditions, for example, in tuberculosis, in which condition a shorter coagulation band is found when there are inflammatory and exudative changes, a longer when fibrotic changes are occurring (Levinson and Klein, 1938, 1939). According to Olhagen (1947), a short band is due to excess of $\alpha$- or $\beta$-globulin whereas a long band reflects an increase in $\gamma$-globulin. This test has been mainly used in conditions other than those involving the liver.

## The Formol Gel Test

This is another test for detecting increase in globulin.

**Technique.** Add 1 drop of formalin to 1 ml. of serum in a narrow test tube. Shake. When positive the serum solidifies within a few minutes, sometimes becoming opaque. A positive test is mainly found in conditions in which there is increased serum globulin. Indeed, the connection with the total globulin appears to be closer than that in the other flocculation tests. Although this test is positive in chronic liver disease, like the Weltmann test it is mainly used in other conditions, such as kala-azar, whilst positive findings have been reported in a number of diseases such as multiple myeloma, sarcoidosis, severe malarial infections, trypanosomiasis and in many chronic infections.

## Summary

Flocculation tests have aroused considerable interest in recent years, both from a clinical standpoint and as regards their theoretical background. A useful summary of some aspects is contained in a paper by Maclagan (1948), who gives the more important references. In liver disease and particularly in acute hepatitis, cephalin cholesterol, colloidal gold and thymol turbidity tests are most useful. The tests are positive in about 90 per cent. of cases of infective hepatitis and cirrhosis, but the percentage of positive results is much lower in arsenical jaundice and in homologous serum jaundice. In

obstructive jaundice, thymol and colloidal gold tests give more consistently negative results than the cephalin test. The cephalin cholesterol test has the advantage of being positive earlier in infective hepatitis, whilst the thymol turbidity remains positive longer.

If one test only is used, the thymol turbidity is best, since the thymol buffer is readily reproducible and the test is most easily read. Some workers prefer to use two and select the thymol turbidity test and the colloidal gold test. Maclagan (1950) finds close correlation between the zinc sulphate and colloidal test and since the zinc sulphate test is simpler prefers to use this.

In interpretating these tests it should be borne in mind that all give positive results in a number of diseases other than those involving the liver, and some, particularly the Weltmann test and Formol Gel test, have been mainly used in such conditions. Positive results in non-hepatic diseases are less frequent in the colloidal gold, cephalin cholesterol and thymol turbidity tests. Carter and Maclagan (1946), using the thymol turbidity test, reported 80 per cent. positive results in malaria, 38 per cent. in rheumatoid arthritis, 36 per cent. in heart failure, 58 per cent. in glandular fever, and 86 per cent. in subacute bacterial endocarditis. These conditions are not likely to be confused clinically with primary liver disorders. The colloidal gold test, which was found to give a higher percentage of positive results in these conditions than the thymol turbidity test, was positive in 94 per cent. of cases of malaria, 76 per cent. of rheumatoid arthritis, 39 per cent. of heart failure, 95 per cent. of glandular fever and 100 per cent. of subacute bacterial endocarditis. Positive results have also been obtained with the cephalin cholesterol test in congestive heart failure, malaria, subacute bacterial endocarditis, infectious mononucleosis, myelomatosis, sarcoidosis and rheumatoid arthritis.

The results of flocculation tests do not always parallel alterations in the albumin-globulin ratio.

## HIPPURIC ACID SYNTHESIS TEST

The best known test based on the *detoxicating function of the liver* is the Hippuric Acid test of Quick. The liver removes benzoic acid by combining it with glycine to form hippuric acid which is excreted in the urine. The benzoic acid is given as sodium benzoate, either by mouth or intravenously, and the amount of hippuric acid excreted in a fixed time is determined. The test thus depends on the ability of the liver to produce sufficient glycine. For reliable results renal function must be normal. If there is any reason to suspect renal impairment carry out a urea clearance test. Both oral and intravenous forms of the hippuric acid test are in use.

### Determination of Hippuric Acid in Urine

The hippuric acid is precipitated from acidified urine by saturation with sodium chloride or ammonium sulphate, is then filtered, washed, dissolved in hot water and the amount determined by titration with sodium hydroxide.

**Reagents.** 1. Sulphuric acid, 25 per cent. (approximately 10 N).
2. Solid sodium chloride.
3. Saturated sodium chloride solution.
4. N/2 sodium hydroxide solution.
5. Barium chloride solution.
6. Phenolphthalein as indicator.

**Technique.** Add 30 grams of sodium chloride to 100 ml. of urine in a suitable flask and shake well to dissolve. Add 3 ml. of the 10 N sulphuric acid. The mixture should be acid to Congo red. Stir with a glass rod, scratching the side of the flask to encourage precipitation of the hippuric acid. Stand at least half an hour, or overnight. Then filter, preferably with the aid of suction through a sintered glass filter. Wash several times with saturated sodium chloride until the filtrate does not give a test for sulphuric acid with barium chloride. Before pouring it on to the precipitate use the wash liquid to wash out the flask in which the precipitation was carried out. Finally, dissolve the precipitate in hot distilled water. The water for this may be heated in the flask used for the precipitation. Titrate with N/2 sodium hydroxide using phenolphthalein as indicator.

Sometimes, particularly with urines containing bile, precipitation may not occur satisfactorily, but may be assisted by seeding with a few small crystals of hippuric acid.

**Calculation.** 1 ml. of sodium hydroxide is equivalent to 0·072 gram of sodium benzoate or 0·061 gram of benzoic acid.

It is necessary to make an allowance for some hippuric acid which remains in solution when the urine is saturated with the sodium chloride. This is done by adding 0·10 gram per 100 ml. of urine. As an example, suppose 7·6 ml. of N/2 sodium hydroxide were required in the titration.

Grams hippuric acid per 100 ml. of urine
$= (7{\cdot}6 \times 0{\cdot}072) + 0{\cdot}1 = 0{\cdot}547 + 0{\cdot}10 = 0{\cdot}65$, expressed as sodium benzoate.

### Technique of the Hippuric Acid Synthesis Test

1. **Oral Test.** Dissolve 6 grams of sodium benzoate in about 200 ml. of water. The test may be begun two to three hours after a breakfast of toast and coffee ; food should not then be given until late in the test. The patient empties the bladder, the urine being discarded, and then drinks the benzoate. The bladder is emptied again four hours later. Any urine passed during this four hours is kept and added to that passed at the end of the four hours. The amount of hippuric acid excreted in this four-hour period is determined.

INTERPRETATION

At least 3 grams of hippuric acid, expressed as benzoic acid, or 3·5 grams as sodium benzoate, should be excreted in health. Smaller amounts are found when there is either acute or chronic liver damage. Amounts lower than 1 gram may be excreted by patients with infective hepatitis.

2. **Intravenous Test.** Because of the possibility of impaired absorption of the benzoate, and also because of the nausea which it sometimes causes when taken orally, an intravenous form of the test has been used. The amount used is 1·77 grams of sodium benzoate dissolved in 20 ml. of water given intravenously. Shortly before the injection the patient empties the bladder and drinks a cupful of water. The bladder is emptied one hour and two hours after the injection. Some workers have used only the single hourly specimen but it would appear to be wiser to collect two specimens.

INTERPRETATION

Hippuric acid equivalent to at least 0·85 gram of sodium benzoate or to 0·7 gram of benzoic acid, should be excreted in the first hour ; or equivalent to 1·15 grams of benzoic acid in the first two hours. As before, smaller amounts indicate the presence of liver damage.

**Note.** If a specimen with a volume less than 100 ml. is obtained it can be diluted to 100 ml. with water and the estimation carried out as described. For very large volumes, more commonly obtained in the oral test, it is advisable to concentrate the urine somewhat before estimating the hippuric acid.

## TESTS BASED ON THE REMOVAL OF INJECTED SUBSTANCES

### The Bromsulphthalein Test

The ability of the liver to excrete certain dyes is used in the bromsulphthalein excretion test (Mateer *et al.*, 1942).

In the original form of this test 2 mg. of dye per kilogram of body weight as a 5 per cent. solution were injected intravenously. This should be almost completely excreted in thirty minutes. The test was later modified to use 5 mg. per kilogram and forty-five minutes used instead of thirty minutes for the time when the dye should have been completely removed from the blood. An intermediate determination at twenty-five minutes may be used to give an additional indication of the rate of removal.

**Technique of the Test.** With the patient fasting, inject intravenously and slowly in one minute an amount of 5 per cent. bromsulphthalein which contains 5 mg. of bromsulphthalein for each kilogram of body weight. Withdraw 5 to 10 ml. of blood twenty-five and forty-five minutes after the injection and allow the specimens to clot. Separate the sera and estimate the amount of dye in each.

### Estimation of Bromsulphthalein

**Reagents.** 1. N/10 sodium hydroxide.

2. N/10 hydrochloric acid.

3. Standard solution of bromsulphthalein. Dissolve 100 mg. of the solid dye in water and make up to 100 ml. Dilute 1 in 10 for use so that the final solution contains 10 mg. per 100 ml.

**Technique.** To 1 ml. of serum add 5 ml. of water and 6 ml. of

N/10 sodium hydroxide. Put up a blank at the same time, using 1 ml. of serum, 5 ml. of water and 6 ml. of N/10 hydrochloric acid. Read in the photoelectric colorimeter using a yellow-green filter, transmission about 540 millimicrons.

Prepare a standard curve as follows :

| Mg. bromsulphthalein per 100 ml. serum | 0 | 2 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|---|
| Ml. standard solution (10 mg. per 100 ml.) | 0·0 | 0·2 | 0·4 | 0·6 | 0·8 | 1·0 |
| Ml. water | 6·0 | 5·8 | 5·6 | 5·4 | 5·2 | 5·0 |

Add 6 ml. of N/10 sodium hydroxide to each, and use the zero tube to set the instrument or to read the others against.

**Calculation.** It may be assumed that 10 mg. of dye per 100 ml. of serum represents the maximum concentration reached. The twenty-five and forty-five minute readings are then expressed as a percentage of this.

INTERPRETATION

In healthy adults not more than 5 per cent. of the dye should remain in the blood at the end of forty-five minutes. The bulk of the dye is removed in twenty-five minutes, less than 15 per cent. being left at the end of that time. Removal proceeds more slowly if liver function is impaired, and in advanced cirrhosis, for example, 40 to 50 per cent. may still be present forty-five minutes after the injection.

Since the dye is removed in the bile, this test can only be used in cases in which there is no obstruction to the flow of bile. It is most useful in liver cell damage without jaundice, in cirrhosis and chronic hepatitis. In these chronic liver conditions it may be the most sensitive of the liver function tests.

## The Bilirubin Excretion Test

The rate at which bilirubin injected intravenously is removed from the blood is studied. Soffer (1933, 1937) used 1 mg. of bilirubin per kilogram of body weight, with a maximum of 70 mg. This was dissolved in 15 ml. of N/10 sodium carbonate. The carbonate was boiled, cooled to 80° C. and the bilirubin dissolved. A specimen of blood (I) was taken, and the bilirubin given intravenously. Two other specimens of blood were then taken from the other arm, one five minutes after the injection (II), the other at the end of four hours (III). The bilirubin content of these three sera was determined, and the percentage bilirubin retained, calculated. This is given by

$$\text{Percentage bilirubin retained} = \frac{\text{Serum (III) bilirubin} - \text{Serum (I) bilirubin}}{\text{Serum (II) bilirubin} - \text{Serum (I) bilirubin}} \times 100.$$

Not more than 5 per cent. of the bilirubin should be retained. The test is most useful in cases of liver disease without jaundice, but has

been used in jaundice (Weech *et al.*, 1941). The bromsulphthalein test is simpler to carry out, is at least as sensitive, and is preferred.

## PROTHROMBIN TIME

Prothrombin time is the term given to the time required for clotting to take place in oxalated plasma to which optimum amounts of thromboplastin and calcium have been added. In these circumstances so long as adequate fibrinogen is present it is held that the clotting time is related inversely to the amount of prothrombin present. This is only strictly true so long as the simple original theory of the mechanism of blood coagulation holds :

prothrombin + thrombokinase + calcium = thrombin.
thrombin + fibrinogen = fibrin.

It is now known that this simple hypothesis is inadequate and that other factors influence the rate of clotting of the plasma. Nevertheless this method is sufficiently reliable for clinical purposes and is widely used. Introduced by Quick (1932, 1939), the method given is the modification due to Biggs and MacFarlane (1949, 1951), to whose papers the reader is referred for a more complete discussion of this subject.

**Reagents.** 1. Sodium citrate, 3·8 per cent. solution, or potassium oxalate, 2 per cent.

2. Thromboplastin. Prepare from fresh human brain. Remove superficial vessels and meninges, and macerate with three to four volumes of acetone in a mortar. Replace the acetone four times. Dry the powder obtained on a suction filter. Keep the powder, when completely free from acetone, in bottles at room temperature. It will keep for three months.

It is important to use the optimum concentration of this thromboplastin. For use suspend 0·5 gram of the dried powder in 5 ml. of 0·85 per cent. saline and incubate for fifteen minutes at 37° C., mixing occasionally. Centrifuge lightly to bring down the coarser particles, remove the supernatant emulsion and prepare several dilutions, 1 in 2, 1 in 5, and 1 in 10 with the saline. Use these to measure the prothrombin time as described below, of normal plasma diluted 1 in 5 with 0·85 per cent. saline. Use the dilution of the emulsion which gives the lowest clotting time. Make this up freshly when required.

3. Calcium chloride, 0·025 M solution. Dissolve 2·775 grams of the anhydrous salt in water and make up to a litre. Standardize. It is also important to use the optimum concentration of calcium chloride. The amount used by Biggs and MacFarlane is given below, but can be determined.

**Technique.** Collect venous blood in a dry syringe and add to 3·8 per cent. sodium citrate or 2 per cent. potassium oxalate in the proportion of nine volumes of blood to one of citrate or oxalate. Mix by inverting several times and centrifuge at 1,500 r.p.m. for fifteen minutes. Remove the plasma and keep in the refrigerator until used.

It is best to do the test within a few hours from taking the blood, since the coagulation time of pathological plasma can alter rapidly on storage. Add 0·1 ml. of undiluted plasma to 0·1 ml. of the emulsion diluted as determined by the method given above. Warm the mixture in a water bath to 37° C. Add 0·1 ml. of the 0·025 M calcium chloride quickly using a graduated Pasteur pipette, and start a stop-watch. Note the time required for coagulation. Carry out the test in triplicate taking the mean of the three readings. Determine the prothrombin time of normal plasma in triplicate in the same way. The normal plasma should clot in less than thirty seconds if the test is to be satisfactory.

**Calculation.** Many authors have used a prothrombin index to express the results.

$$\text{Prothrombin index} = \frac{\text{Prothrombin time of control plasma}}{\text{Prothrombin time of unknown}} \times 100.$$

**Note.** The range of optimum concentrations of calcium chloride and thromboplastin are narrower for abnormal than for normal plasmas.

INTERPRETATION

Although determination of prothrombin time has been most used in controlling dicoumarol and tromexan therapy, it has also been used in liver disease and jaundice. Prothrombin is formed by the liver cells, vitamin K being required. When bile salts are not present in the intestine, the absorption of vitamin K from the intestine is impaired. Accordingly, in jaundice and liver cell disease, the amount of prothrombin formed may be reduced:

1. Because there is defective absorption of vitamin K. This occurs in obstructive jaundice when there is absence of bile salts from the intestine.

2. Because there is liver cell damage. The liver is then less able to form prothrombin even though absorption of vitamin K may be normal.

It is thus seen that the formation of prothrombin is impaired both in obstructive jaundice and in diseases in which there is liver cell damage, so that the prothrombin time cannot be used to differentiate between them. However, if adequate vitamin K is given parenterally the prothrombin time returns rapidly to normal in uncomplicated obstructed jaundice, whereas in liver damage the response is less marked. Investigation of the effect of vitamin K dosage on the prothrombin time has been used as a test for impaired liver function. One method of carrying out such an investigation is to determine the prothrombin index during the morning, shortly before giving 200 mg. of synkevit (2 methyl 1·4 naphthohydroquinone diphosphate) intravenously. Give a further intravenous injection of 100 mg. six hours later, and repeat the prothrombin determination next morning, twenty-four hours after the first injection. A marked increase in prothrombin index results if the liver function is normal.

Determination of prothrombin time is also used to decide whether

there is a danger of bleeding at operation in biliary tract disease. In normal persons the prothrombin index should be within the range 90 to 100 per cent. of the normal controls used. The critical level below which bleeding may occur is not a fixed one, but usually lies below a value between 20 and 40 for the prothrombin index.

## MISCELLANEOUS DETERMINATIONS

### Serum Alkaline Phosphatase in Liver Disease and Jaundice

This enzyme is more fully discussed elsewhere (see p. 313), but some reference must be made here to its use in jaundice. Serum alkaline phosphatase is increased in both acute hepatitis and obstructive jaundice. However, the rise is usually much greater in cases of obstructive jaundice than in jaundice due to hepatitis. Using King-Armstrong units (p. 311), the dividing line is often put at about 35 units per 100 ml. of serum. A value higher than this suggests obstructive jaundice, in which condition very high figures up to 200 units and over, may be found. On the other hand results below 35 units suggest conditions such as infective hepatitis. There is a certain amount of overlapping, almost entirely in the range 25 to 45 units per 100 ml. In addition, very high values are occasionally obtained in liver disease, for example, in xanthomatous biliary cirrhosis, in which there is no extrahepatic obstruction. Nevertheless the test is valuable, particularly when taken in conjunction with other tests such as the thymol turbidity test (see later, p. 258). Serum alkaline phosphatase is normal in hæmolytic jaundice.

The explanation of these increases is not clear. That they are due to the fact that the enzyme is excreted in the bile is not adequate to explain all the observed facts. Production of the enzyme by the liver may be a further factor.

### Serum Choline Esterase

**Determination, Method of Stedman et al., (1933)**

The acetic acid liberated by the action of the enzyme on acetylcholine is titrated with sodium hydroxide.

**Reagents.** 1. Acetylcholine substrate. Dissolve 1 gram of acetylcholine bromide in 10 ml. of water.

2. Cresol red indicator.
3. Sodium hydroxide, N/10.
4. Borate buffer, *p*H 8 (see p. 516).
5. Sodium hydroxide.

**Technique.** In a large test tube in a water bath kept at 30° C. place some freshly distilled carbon dioxide-free water. Add 1 ml. of the cresol red and 1·5 ml. of acetylcholine substrate. Adjust to *p*H 8 with a few drops of N/10 sodium hydroxide, comparing with a tube containing 50 ml. of borate buffer and 1 ml. of cresol red, tightly corked and also placed in the water bath. Now add 0·5 ml. of serum to the substrate, again adjust to *p*H 8, and stopper the tube with a rubber bung through which passes an intravenous needle connected

by fine rubber tubing to a burette containing N/100 sodium hydroxide. Add alkali drop by drop, shaking constantly, to neutralize the acetic acid formed and so keep the *p*H constant at 8. Read the burette at the end of twenty minutes.

**Calculation.** The result is expressed directly in ml. of N/100 sodium hydroxide required. It was found that a blank determination without serum required 0·1 ml. of N/100 sodium hydroxide to keep the *p*H at 8. Subtract this from each reading.

INTERPRETATION

The normal range by the above method is 2 to 4 units. Findings in disease are summarized by Augustinsson (1948) in a monograph on this enzyme, but are not very typical. Thus low values have been found for example, in anæmia, tuberculosis, carcinoma, uræmia, and shock, increases in hyperthyroidism and diabetes. Low values have also been reported in liver disease by McCardle (1940) using a method which measures, in a Barcroft manometer, the volume of carbon dioxide liberated by adding acetylcholine to a mixture of serum and Ringer's solution (Jones and Tod, 1935). The acetic acid formed reacts with bicarbonate present in the Ringer's solution.

## The Use of Liver Function Tests

The tests described may be used :

1. In the differential diagnosis between the different types of jaundice.

2. To assess the severity of liver damage in known liver disease, to follow the trend of the disease, to gauge pre-operative surgical risk.

3. As an aid in diagnosing the presence of latent liver disease such as in the differential diagnosis of hæmatemesis or ascites. This includes the assessment of residual damage after recovery from liver disease ; and the screening of suspected cases during outbreaks of infective hepatitis and of persons working in industrial concerns where substances toxic to the liver are being handled.

1. **Differential Diagnosis in Jaundice.** The distinction is not always easy between an obstructive jaundice, particularly one due to a malignant growth, and an infective or toxic hepatitis. To differentiate the two conditions the combination of a flocculation test and serum alkaline phosphatase suggested by Maclagan, is valuable. A normal thymol turbidity test and a high alkaline phosphatase above 50 units is almost diagnostic of obstructive jaundice, whilst a raised thymol turbidity with an alkaline phosphatase say below 30 units, is equally almost diagnostic of infective or toxic hepatitis type of condition. Maclagan claims that these two tests will diagnose almost 80 per cent. of cases. If doubt is still felt, further tests made are based on the fact that liver function is normal for at least several weeks in obstructive jaundice, whereas liver cell damage is present from the beginning in infective hepatitis and shows a tendency to improve in most cases. Consequently, during the early stages, tests of liver function will be found normal in obstructive jaundice but impaired in hepatitis. Tests recommended for this purpose are the

galaetose toleranee test and the hippurie aeid synthesis test. Further useful information as to the pateney of the ducts may be gathered from the fæcal and urinary urobilinogen. The qualitative Van den Bergh reaetion is of no value in the differential diagnosis of these eases of jaundice since it is immediate direct in both obstructive jaundiee and in aeute hepatitis. The degree of jaundice can be followed by serial estimations of serum bilirubin. Beyond this it should not be neeessary to go. Plasma proteins are almost always normal in the earlier stages of both groups of eonditions, though the albumin may be low in the normal range. Serum eholesterol is raised in obstruetive jaundiee but more variable in hepatie jaundiee. This and the proportion of ester cholesterol has been diseussed previously (see p. 189).

2. **Assessing the Amount of Liver Damage.** Tests whieh assess some metabolie or other funetion of the liver are ehosen. The galactose tolerance and hippuric aeid synthesis tests are partieularly useful in the presenee of jaundice ; if jaundiee is absent, the bromsulphthalein test should also be used. The estimation of serum proteins is also useful. The fall in serum albumin and the rise in serum globulin whieh may oceur give an indieation of the severity of the condition and are valuable both in diagnosis and prognosis.

3. **As an Aid in Diagnosing Latent Liver Disease.** The liver has considerable funetional reserve and eapaeity for regeneration in ehronie liver disease, for example, in alcoholie eirrhosis. The problem is mainly to decide whieh of the tests gives the most sensitive indieation of impaired liver funetion. Sinee there is not a uniform failure of the various liver funetions, some are still normal while others are impaired. So in testing for latent liver disease it is better to use several of the tests than to rely on any one. In most of these eases of suspected liver disease, jaundiee will be absent, so that the bromsulphthalein test ean almost always be used and on the whole it is the most sensitive of the tests at present available. If this, the galaetose toleranee test, the hippurie aeid test, and urine urobilinogen are all normal, it is doubtful whether any tests now available would reveal the presence of liver damage.

## Summary

A useful eombination of tests which ean be done on a single speeimen of elotted blood, is : serum bilirubin, one or more floeeulation tests (for example, thymol turbidity test, zine sulphate test), serum alkaline phosphatase, and serum albumin and globulin. Examination of urine (and sometimes fæees) for the amount of urobilinogen present ean be done without any further trouble to the patient and may yield useful information. Additional tests need only be done on patients in whom the diagnosis is still not elear.

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## *CHAPTER XV*

# TESTS OF PANCREATIC FUNCTION. STEATORRHŒA

Changes in both the internal and external secretions of the pancreas may be found in pancreatic disease.

As regards the *internal secretion*, insulin, conditions in which the islets of Langerhans are affected whilst the external secretion is normal have already been considered. Glycosuria with some degree of hyperglycæmia is sometimes found in acute pancreatitis. Since considerable recovery of insulin function takes place, complete restitution occurs in most cases but occasionally a permanent diabetes mellitus is produced when pancreatic necrosis has occurred. Diabetes mellitus is likely to be present after recurring attacks and in the later stages of chronic pancreatitis. However, tests in pancreatic disease are mostly concerned with the external secretion of the organ.

The *external secretion* into the intestine, containing sodium bicarbonate and the enzymes, amylase, lipase, and the group of proteinases still often referred to as trypsinogen, is controlled by a dual mechanism. Secretin elaborated in the duodenal mucosa when acid foodstuffs pass from the stomach into the duodenum, is responsible for the output of water and bicarbonate, whilst pancreozymin, also from the duodenal mucosa, on injection intravenously, increases the output of enzymes. Vagal stimulation also increases the output of enzymes.

Pancreatic function can be studied *directly* by quantitative estimation of the output of fluid, bicarbonate, and enzymes obtained by duodenal intubation and following pancreatic stimulation. This method, though accurate, is laborious and time consuming so that *indirect* methods are more often used. These may be divided into two groups.

(*a*) *Determination of Enzymes in Serum and Urine.* In acute pancreatitis or when the pancreatic duct is obstructed, amylase and lipase may be absorbed into the portal blood stream and be found in increased quantities in the peripheral blood. In chronic pancreatitis and carcinoma of the pancreas the serum enzymes are less frequently raised, but attempts have been made to increase the sensitivity of the tests in these diseases by stimulating the gland and thus increasing the output of the enzymes. Urinary amylase in most conditions is increased when the serum amylase is increased.

(*b*) *Study of the Fæces.* In severe pancreatic deficiency evidence of impaired digestion of foodstuffs may be obtained from the stools, which become fatty and contain excess nitrogen. Quantitative estimation of the average daily excretion of fat and nitrogen in the fæces are made when the patient is given a standard diet containing known amounts of fat and protein.

## EXAMINATION OF DUODENAL CONTENTS FOLLOWING STIMULATION OF THE PANCREAS

The secretory powers of the pancreas can be studied by collecting duodenal contents after an injection of secretin. For this purpose Ågren and Lagerlöf (1936 ; see also Lagerlöf, 1939, 1942) use a tube with a double lumen, one part of which is 10 inches longer than the other. The end of the shorter tube is in the stomach, that of the longer tube in the duodenum.

**Insertion of the Tube and Collection of Specimens** (Lagerlöf, 1942). With the patient in a sitting position place the end of the tube at the back of the tongue. Press this with a spatula so that the end enters the œsophagus. Push the tube down into the stomach, which is entered at 45 to 50 cm. from the teeth. At 70 cm. the end will reach to the region of the pylorus. Place the patient on the right side and introduce a further 10 cm. The time required for the tube to pass through the pylorus may be anywhere from a half to several hours. When in position, clear, alkaline bile-stained juice should be obtained from the duodenal tube, and juice free from bile from the portion of the tube in the stomach. X-rays can be used to confirm the position of the tube. Duncan *et al.* now use radio opaque tubes and pass under fluoroscopic control. This is usually easier and less uncomfortable for the patient.

Place the patient comfortably in an arm-chair or propped up in bed. Using a water vacuum pump, apply continuous suction, kept constantly at 20 to 30 mm. Hg by means of a mercury trap which lets in air when the suction exceeds this. Collect the duodenal juice in ice-cooled flasks containing glycerol. Shake now and then to mix. An amount of glycerol equal to the volume of juice is used to preserve the tryptic and lipolytic activity. Remove saliva continuously by suction from the mouth. Use a dose of one clinical unit of secretin per kilogram of body weight given intravenously (1 clinical unit = 16 cat units. 1 mg. = 253 cat units). After the injection, collect specimens first for two ten-minute periods, then for two twenty-minute periods. Following the injection of secretin there is a marked increase in the volume of fluid coming from the duodenum. When the gall-bladder is normal, the second or third samples may contain no bile, but bile-staining persists throughout the test when the gall-bladder function is impaired by cholecystitis. Absence of bile pigment from all specimens indicates obstruction of the bile ducts.

It is usual to measure the volume and to determine the bilirubin and bicarbonate content and the amylase, trypsin, and lipase activities. Since other workers have based their technique on the work of Ågren and Lagerlöf, their methods are still used and several will be given.

### Examination of Specimens

**Bicarbonate.** The Van Slyke apparatus used for determining alkali reserve (see p. 354) can be used. This is the more satisfactory

method. Lagerlöf measures the bicarbonate by indirect titration with hydrochloric acid. Add 10 ml. of N/10 hydrochloric acid to 5 ml. of duodenal contents, boil, cool, and titrate with N/10 sodium hydroxide using phenolphthalein as indicator. The difference between the volume of acid added and the volume of alkali used gives a measure of the amount of bicarbonate present. Express the result in terms of milli-equivalents (see p. 347).

**Bilirubin.** Meulengracht's method can be used. Dilute the duodenal juice with 0·9 per cent. sodium chloride until it matches a 1 in 10,000 solution of potassium dichromate as described for determining the icteric index of serum (p. 230).

### Determination of the Lipase Activity of Duodenal Contents

Lagerlöf used a viscosimetric method, but the American workers have mainly used the method of Cherry and Crandall (1932) as modified by Comfort and Osterberg (1934), given later for blood (p. 274). Diamond and his co-workers, whose figures are given later, used duodenal juice diluted 1 in 10, otherwise proceeding as described for blood.

### Determination of Trypsin in Duodenal Contents

**Method of Lagerlöf** (1942). Casein is digested by the duodenal juice at a *p*H of 9 and at 30° C. and the increase in carboxyl groups determined by titration with potassium hydroxide. Ninety per cent. alcohol is added and in its presence the basic properties of the amino groups are suppressed so that the titration can be done directly.

**Reagents.** 1. Casein solution. Shake 30 grams of casein vigorously with 300 ml. of water for five minutes. Then add 20 ml. of normal ammonium hydroxide and shake the mixture for fifteen to twenty minutes, pour into a volumetric flask and make up to 500 ml. In the refrigerator the solution keeps at least four days.

2. Buffer solution. Mix 120 ml. each of normal ammonium chloride solution and normal ammonia, and add 160 ml. of distilled water.

3. N/10 alcoholic potassium hydroxide. Mix 1 part of normal potassium hydroxide with 9 parts of absolute alcohol.

4. Thymolphthalein. Use a 0·5 per cent. solution in 95 per cent. alcohol.

5. Ethyl alcohol, 95 per cent.

**Technique.** Take two 250 ml. conical flasks and measure into each 38 ml. of 95 per cent. alcohol, 3 ml. of water and 1 ml. of indicator. Measure 2 ml. of duodenal juice (1 : 1 with glycerol, kept in the refrigerator) into a test tube, allowing the pipette to drain thoroughly. Place the tube in a water bath at 30° ± 0·1° C. and five and a half minutes later add 2 ml. of buffer solution, and after exactly thirty seconds add 3 ml. of casein solution. Shake well. Exactly twenty minutes after adding the casein, pour the contents of the tube into one of the conical flasks, rinsing the tubes out with the contents of the flask. Allow the tube to drain completely into the flask. This terminates the reaction. Place the same amount of duodenal juice,

buffer, and casein in the other flask. Titrate both flasks with the potassium hydroxide to the first appearance of a blue colour. Add 72 ml. of boiling 95 per cent. alcohol to each flask. The blue colour disappears. Continue the titration to the same colour in both flasks. The difference between the two titrations gives a measure of the amount of tryptic activity. For satisfactory results it is necessary to work rapidly.

**Calculation.** Units of trypsin are related to the difference between the ml. titration of test and the ml. titration of blank, as follows :

| Ml. N/10 potassium hydroxide | Units trypsin | Ml. N/10 potassium hydroxide | Units trypsin | Ml. N/10 potassium hydroxide | Units trypsin |
|---|---|---|---|---|---|
| 0·10 | 0·037 | 0·80 | 0·335 | 1·50 | 0·78 |
| 0·20 | 0·075 | 0·90 | 0·385 | 1·60 | 0·87 |
| 0·30 | 0·115 | 1·00 | 0·44 | 1·70 | 0·97 |
| 0·40 | 0·157 | 1·10 | 0·50 | 1·80 | 1·07 |
| 0·50 | 0·200 | 1·20 | 0·56 | 1·90 | 1·18 |
| 0·60 | 0·245 | 1·30 | 0·63 | 2·00 | 1·28 |
| 0·70 | 0·290 | 1·40 | 0·70 | | |

**Gowenlock's Method** (Gowenlock, 1953)

Gowenlock has devised a method similar to that of Hunt for pepsin, using denatured dried serum proteins as substrate and measuring the amount of activity by means of the colour given with Folin and Ciocalteu's reagent. This method is preferred to the above Lagerlöf method.

**Reagents.** 1. Trichloracetic acid, 6 per cent. (w/v) solution.

2. Substrate. Dissolve 4 grams of dried serum in 100 ml. of N/100 sodium hydroxide in a flask plugged with cotton wool. The *p*H is approximately 10. Immerse in boiling water for ten minutes. Cool to room temperature under running water and filter through cotton wool. Add one quarter of its volume of ammonium chloride—ammonia buffer prepared by adding enough ammonia, S.G. 0·88, to bring a molar ammonium chloride solution to *p*H 9·5.

3. N/4 sodium hydroxide.

4. Folin and Ciocalteu's reagent (see p. 310).

5. Standard phenol solutions containing 10, 20, 30, 40, 50, 60, and 70 mg. per litre of N/1,000 hydrochloric acid.

**Technique.** Set up four tubes, (1) and (2) containing 5 ml. of buffered substrate, (3) and (4) containing 10 ml. of 6 per cent. trichloracetic acid. Bring these and the duodenal juice—glycerol mixture (50 per cent. v/v) to 37° C. in a water bath. At zero time add 1 ml. of juice to (1) and thirty seconds later add 1 ml. of juice to tube (4). Mix. After fifteen minutes incubation mix (1) and (3), the test, and (2) and (4) the control, pouring the contents backwards and forwards in order to mix thoroughly. Leave in the bath for four to five minutes. Filter through a 12·5 cm. No. 1 Whatman paper (refilter through a No. 30 if the filtrate is a little cloudy).

Measure 2 ml. of the filtrate into a 100 ml. conical flask containing 20 ml. of N/4 sodium hydroxide, mixing whilst running in. Follow with 1 ml. of Folin and Ciocalteu's reagent. After thirty minutes read the intensity of the blue colour in the photoelectric colorimeter using a red filter or transmission at 680 millimicrons. The difference between the test and the control gives a measure of the tryptic activity. Prepare a standard curve by using 2 ml. of the standard phenol solutions instead of 2 ml. of the protein-free filtrate. One unit of tryptic activity is present when 1 ml. of the juice-glycerol mixture gives a protein-free filtrate with a chromogenic activity equal to that of a phenol solution containing 1 mg. of phenol per litre.

Gowenlock found that 1 Lagerlöf unit by the previous method corresponds to 70·5 units by his method.

**X-ray Film Method** (Gordon *et al.*, 1952 ; Harrison, 1947)

It will be convenient to include this useful approximate method here. Prepare serial dilutions of duodenal juice as follows. Place 0·2 ml. of 1 per cent. crystalline sodium carbonate solution in each of twelve test tubes. Add 0·2 ml. of juice to the first. Mix well and transfer exactly 0·2 ml. to the second tube, and so on to tube six. To the seventh tube add 0·2 ml. of duodenal juice diluted 1 in 64 with the carbonate solution, and proceed as before. Then, beginning with the highest dilution, place a drop of each mixture on to an X-ray film, delivering this in such a way that each drop occupies the same area of film. Place 1 drop of the carbonate on as a control. Put the film into a covered Petri dish which contains a moist pad of cotton wool to prevent drying, and incubate for thirty minutes at 37° C. Transfer to the freezing chamber of the refrigerator for twenty minutes. Then wash the film in cold water. Digestion is shown by a clear translucent area, whereas there is no change other than some roughness where there has been no digestion. Read the highest dilution which shows a completely translucent area.

This method is useful for examining duodenal juice obtained from children. Normal juice showed digestion up to tube (6) or beyond, whereas in fibrocystic disease of the pancreas digestion did not proceed beyond tube (2).

**Application to Fæces.** This technique can be used to show the presence of trypsin in the stools. Make a suspension of fæces in the carbonate solution of 1 in 4, and put up serial dilutions as described above.

**Determination of Amylase in Duodenal Contents**

Method of Norby (1935) adapted by Lagerlöf (1942).

Starch is digested and the amount of maltase formed is estimated by the blood sugar method of Hagedorn and Jensen.

**Reagents.** 1. Sodium chloride. 1·5 per cent. solution.

2. Starch solution. Mix 5 grams of dried soluble starch with a little distilled water, and add 200 ml. of boiling distilled water. Add 250 ml. of a phosphate buffer prepared by mixing equal quantities of M/15 disodium hydrogen phosphate, $Na_2HPO_4 2H_2O$, and M/15

potassium dihydrogen phosphate, transfer to a 500 ml. flask and make up to the mark with water.

3. N/10 sodium hydroxide.

4. Alkaline potassium ferricyanide. Dissolve 8·3 grams of potassium ferricyanide and 10·6 grams of anhydrous sodium carbonate in water and make up to 500 ml. This solution is N/20.

5. Acetic acid, 50 per cent. (v/v).

6. Iodide, chloride, sulphate solution. Twenty-five grams potassium iodide, 250 grams sodium chloride, 50 grams of zinc sulphate, $ZnSO_4 . 7H_2O$, per litre (see p. 40).

7. N/20 sodium thiosulphate.

8. Starch indicator.

**Technique.** Dilute the duodenal juice with 1·5 per cent. sodium chloride to 1 in 800 or 1 in 400 (usually the latter for the second and third specimens). Measure 2 ml. of the diluted juice into a test tube (Hagedorn tube) and heat for eight minutes at 37° C. in a water bath. Noting the time, add 2 ml. of the starch solution, also previously warmed to 37° C. After exactly fifteen minutes, pipette 2 ml. into a tube containing 1 ml. of N/10 sodium hydroxide. This stops the digestion. Add 2 ml. of alkaline potassium ferricyanide and 12 ml. of distilled water and place in a boiling water bath for twenty minutes. Cool immediately to room temperature, add 2 ml. 50 per cent. acetic acid and 10 ml. of the iodide, chloride, zinc sulphate solution. After at least three minutes, titrate with N/20 sodium thiosulphate. As a control, measure 1 ml. of N/10 sodium hydroxide into a similar tube, add 1 ml. of diluted duodenal juice and 1 ml. of the warmed starch solution, and proceed as described for the test.

**Calculation.** The difference between the two titrations shows how much N/20 potassium ferricyanide has been reduced by the maltose

TABLE XV.

*Conversion of ml. N/20 $K_3Fe(CN)_6$ into units Amylase per ml. of Duodenal Juice, using a* 1 *in* 1,000 *Dilution*

| Ml. N/20 ferricyanide | Units per ml. | Ml. N/20 ferricyanide | Units per ml. |
|---|---|---|---|
| 0·05 | 0·43 | 0·75 | 7·01 |
| 0·10 | 0·86 | 0·80 | 7·56 |
| 0·15 | 1·28 | 0·85 | 8·10 |
| 0·20 | 1·72 | 0·90 | 8·65 |
| 0·25 | 2·15 | 0·95 | 9·21 |
| 0·30 | 2·60 | 1·00 | 9·81 |
| 0·35 | 3·06 | 1·05 | 10·41 |
| 0·40 | 3·53 | 1·10 | 11·03 |
| 0·45 | 4·00 | 1·15 | 11·63 |
| 0·50 | 4·47 | 1·20 | 12·26 |
| 0·55 | 4·96 | 1·25 | 12·90 |
| 0·60 | 5·46 | 1·30 | 13·55 |
| 0·65 | 5·97 | 1·35 | 14·23 |
| 0·70 | 6·49 | 1·40 | 14·92 |

(*From Acta medica Scandinavica, Dr. Lagerlöf.*)

formed. From this volume the units of amylase per ml. of duodenal juice are calculated. One ml. of the ferricyanide equals 2·15 mg. of maltose, and is equivalent to 1 ml. of N/20 thiosulphate.

Make the necessary adjustment in the calculation if a different dilution is used.

INTERPRETATION

The ranges obtained in normal cases are as follows (see Diamond and co-workers, 1939, 1940, 1941 ; Comfort and Osterberg, 1941 ; Dornberger *et al.*, 1948 ; see also Howat, 1952) :

| | |
|---|---|
| Volume . . . | 130 to 250 ml. (2·1 to 4·5 ml. per kg. per hour). |
| Bicarbonate . . | 90 to 130 milli-equivalents. |
| Amylase. . . | 250 to 1,200 units (5·5 to 11 units per kg. per hour). |
| Trypsin . . | 20 to 40 units (0·35 to 0·7 units per kg. per hour). |
| Lipase . . . | 7,000 to 14,000 units (135 to 225 units per kg. per hour). |

The rise in bicarbonate concentration is roughly proportional to the increase in the rate of secretion although it occurs a little later, whereas the opposite is the case with the enzymes whose concentration varies inversely with the rate of secretion. The curves of enzyme secretion are roughly parallel with each other. In considering the results it is necessary to calculate the actual amounts secreted from the concentration and the total volume.

Whereas the volume of juice and the amount of bicarbonate produced is proportional to the amount of secretin given, the amounts of the enzymes are independent of this.

In disease, findings may be broadly divided into two groups though intermediate results are obtained.

1. A fall in bicarbonate and enzymes either points to duct obstruction or shows a reduction in the amount of secreting tissue such as may occur in acute necrosis or malignant growth of the pancreas. The fall in volume may not be so marked in pancreatitis since gall-bladder function may be impaired and allow bile to flow continuously into the intestine. In carcinoma of the head of pancreas which obstructs both common bile duct and pancreatic duct, volume, bicarbonate and enzymes are all reduced.

2. In milder forms of pancreatitis and following acute pancreatitis, dissociated findings are obtained. Volume and bicarbonate are fairly normal, but enzyme secretion especially that of amylase and to a lesser extent of lipase, is reduced. Trypsin is less affected and appears to be normal in the majority of cases.

## TESTS BASED ON THE DETERMINATION OF ENZYMES IN SERUM AND URINE

### Determination of Serum Amylase

#### I. Saccharogenic Method of Somogyi (Somogyi, 1938)

Serum is incubated with a starch solution and the amount of reducing substance present is determined before and after the incubation. The difference gives a measure of the amylolytic activity of the serum.

**Reagents.** 1. Starch solution. Suspend for about an hour, with frequent shaking, 100 grams of starch in a litre of approximately N/100 hydrochloric acid. Allow to settle, pour off the acid and add a litre of 0·05 per cent. sodium chloride solution. Stir well, allow to settle, decant, and repeat the washing with the salt solution. Then spread out the starch and allow it to dry in air.

Grind thoroughly 1·5 grams of this washed starch in a mortar with a few ml. of water and add to about 70 to 80 ml. of boiling water. Rinse out the mortar with a few ml. portions of water and add to the boiling starch solution. Boil for about a minute after the last addition of water and then heat in a water bath for fifteen to thirty minutes keeping the mouth of the flask covered with an inverted beaker. Allow to cool and make up to 100 ml. with water. This solution will keep for a few days in the refrigerator. Make it up freshly at frequent intervals.

3. Acidified sodium chloride solution. Dissolve 10 grams of sodium chloride in water, add 3 ml. of N/10 hydrochloric acid, and make up to a litre with water.

3. Copper sulphate, 5 per cent. ($CuSO_4$, $5H_2O$) solution.

4. Sodium tungstate, 6 per cent. ($Na_2WO_4$, $2H_2O$) solution.

5. Somogyi's high alkalinity copper reagent. A high alkalinity is used because maltose has a lower reducing power than glucose. Dissolve 25 grams of anhydrous sodium carbonate and 25 grams of Rochelle salt in about 600 ml. of water, add 40 ml. of normal sodium hydroxide, followed by 6 grams of copper sulphate ($CuSO_4$, $5H_2O$) dissolved in about 100 ml. of water and added with constant stirring. Then add 5 grams of potassium iodide and 15 ml. of normal potassium iodate and shake well. When dissolved, make to 1 litre and mix.

6. Sulphuric acid, 5 N.

7. N/200 sodium thiosulphate. Prepare freshly for use from stock N/10 solution.

8. Starch solution as indicator.

**Technique.** Pipette 5 ml. of the starch solution and 2 ml. of the sodium chloride solution into each of three test tubes. Place two of these in a water bath at 40° C. for a few minutes to warm up and then to one add 1 ml. of serum (the test), and to the other 1 ml. of water (the water blank). Then incubate for thirty minutes. For the serum blank add 1 ml. of serum to the third tube followed immediately by 1 ml. of 5 per cent. copper sulphate solution and 1 ml. of sodium tungstate. At the end of the half hour's digestion, remove from the bath and add, with shaking, 1 ml. each of these same solutions. Stand for a few minutes and then centrifuge. Filter the supernatant fluids through small filter papers.

To 5 ml. of each of the supernatant fluids add 5 ml. of the alkaline copper solution and heat in a bath of boiling water for twenty minutes, plugging the tubes lightly with cotton wool. Cool under running water. Add 1 ml. of 5 N sulphuric acid and titrate with N/200 thiosulphate. Since there is excess starch present, a blue colour is formed immediately on acidification, so that as the end-point is approached it is necessary to shake vigorously after each

addition of thiosulphate so as to prevent possible overshooting of the end-point. Titrate 5 ml. of the copper reagent after adding 1 ml. of the sulphuric acid, adding starch for the end-point. About 15 ml. are required.

**Calculation.** The content of reducing substance in the three titrations, expressed as glucose, is read from Table XVI and the amylase calculated as follows :

TABLE XVI

*Determination of Serum Amylase. Somogyi Saccharogenic Method*

Mg. glucose per 100 ml.

| Ml. N/200 $Na_2S_2O_3$ | 0 | 0·1 | 0·2 | 0·3 | 0·4 | 0·5 | 0·6 | 0·7 | 0·8 | 0·9 |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | — | — | — | — | 18 | 22 | 26 | 29 | 32 | 35 |
| 1 | 39 | 42 | 45 | 48 | 52 | 55 | 58 | 61 | 65 | 68 |
| 2 | 71 | 74 | 78 | 81 | 84 | 87 | 90 | 93 | 96 | 99 |
| 3 | 102 | 106 | 109 | 112 | 115 | 118 | 121 | 124 | 127 | 130 |
| 4 | 133 | 137 | 140 | 143 | 146 | 149 | 152 | 155 | 158 | 161 |
| 5 | 164 | 168 | 171 | 174 | 177 | 180 | 183 | 186 | 189 | 192 |
| 6 | 195 | 199 | 202 | 205 | 208 | 211 | 214 | 217 | 220 | 223 |
| 7 | 226 | 229 | 232 | 235 | 238 | 241 | 244 | 247 | 250 | 253 |
| 8 | 256 | 260 | 263 | 266 | 269 | 272 | 275 | 278 | 281 | 284 |
| 9 | 287 | 290 | 293 | 296 | 299 | 302 | 305 | 308 | 311 | 314 |
| 10 | 317 | 321 | 324 | 327 | 330 | 333 | 336 | 339 | 342 | 345 |
| 11 | 348 | 352 | 355 | 358 | 361 | 364 | 367 | 370 | 373 | 376 |
| 12 | 379 | 383 | 386 | 389 | 392 | 395 | 398 | 401 | 404 | 407 |
| 13 | 410 | 414 | 417 | 420 | 423 | 426 | 429 | 432 | 435 | 438 |
| 14 | 441 | 445 | 448 | 451 | 454 | 457 | 460 | 463 | 466 | 469 |
| 15 | 472 | 475 | 478 | 481 | 484 | 487 | 490 | 493 | 496 | 499 |
| 16 | 502 | 506 | 509 | 512 | 515 | 518 | — | — | — | — |

Serum amylase in units per 100 ml.

= Mg. glucose per 100 ml. test − mg. glucose per 100 ml. serum blank − mg. glucose per 100 ml. water blank.

The water blank should be negligible.

**Notes.** 1. For values over 300 units per 100 ml., dilute the serum with 0·9 per cent. saline and repeat the test.

2. Whilst this is, on the whole, the most satisfactory type of method, it is not suitable for cases in which there is a high fasting blood sugar. However, the test can then be done using diluted serum and an appropriately longer digestion time so as to compensate for the dilution.

3. The method can be adapted for use with many of the other commonly used blood sugar methods given earlier (see Chapter II), but it should be noted that slightly different results may be obtained with other alkaline copper reagents.

As an example, with Maclean's method (p. 39), mix 2·5 ml. of the starch solution prepared as above, 1 ml. of the acidified sodium chloride, and 0·5 ml. serum. Immediately withdraw 1·6 ml. and add to 22·4 ml. of the acidified sodium sulphate solution. Incubate

the remainder for thirty minutes, and then again transfer 1·6 ml. to 22·4 ml. sodium sulphate. Bring each of these to the boil, add dialyzed iron and carry out the determination as previously described. The difference between the two results gives the serum amylase in units per 100 ml. of serum. Again, when titrating run the thiosulphate in slowly whilst shaking vigorously.

**Normal Range.** This was given by Somogyi as 80 to 180 units per 100 of serum, but it has been found to be lower than this by some workers, for example Andersch (1946), and this has been the author's experience. A range of from 40 to 120 is suggested.

### II. Somogyi's Iodine Test Method (Somogyi, 1938)

The time required for the complete digestion of a certain amount of starch is determined by periodic testing with iodine.

**Reagents.** 1. *Starch Solution.* This contains 75 mg. of starch and 250 mg. of sodium chloride per 100 ml. of solution. Prepare a stock solution from 1·5 grams of starch as described above, adding 10 ml. of 2·5 per cent. sodium chloride solution before finally making up to 100 ml. with water. Dilute 5 ml. of this to 100 ml. with 0·25 per cent. sodium chloride to obtain the solution for use.

2. *Iodine Solution.* 0·002 N aqueous iodine. Dilute 10 ml. of 0·1 N iodine to 500 ml. with 2 per cent. potassium iodide.

**Technique.** Pipette 4 ml. of the starch solution (containing 3 mg. starch) into a test tube which is placed in a water bath for a few minutes so as to reach 37° C. Add 1 ml. of serum and start the stopwatch. Place 0·5 ml. portions of the iodine solution in test tubes. After five minutes incubation withdraw 0·5 ml. of the mixture and add to one of these test tubes. Withdraw further 0·5 ml. portions at intervals of two minutes and add to further portions of iodine. Continue this until the red-brown colour of erythrodextrin is seen with a barely perceptible tinge of purple. With experience the length of time to be allowed between successive testings can be assessed and the number reduced and the time between the last two narrowed down. It is often possible to reduce the quantities of starch solution and serum to 2 ml. and 0·5 ml. respectively. If digestion is complete on the first test the serum should be diluted. If this is done more than twofold, use less iodine—0·3 ml.

**Calculation.** The formula :

$$\text{Serum amylase (units per 100 ml.)} = \frac{1{,}600}{t\,v} \text{ is used.}$$

$t$ = the time required for the digestion.

$v$ = volume of serum in the mixture, i.e. 1 ml. normally, but less if a dilution is used, for example, 0·2 ml. with 1 in 5 dilution.

Using this formula the values obtained are in close agreement with those of the saccharogenic method, given by Somogyi as 80 to 180 units per 100 ml. serum.

*Other Methods.* Wohlgemuth used a method in which equal amounts of starch solution are added to serial dilutions of serum and after digesting for a fixed time iodine is added to the tubes to find the

tube in which complete digestion has just occurred as in the method for urine given below. Huggins and Russell (1948) use a colorimetric method which measures the amount of blue colour given with iodine by the starch remaining after the digestion. This method has been modified by van Loon, Likins, and Seger (1952). A viscosimetric method has also been used (see Somogyi, 1938).

### Determination of Urinary Amylase (or Diastase)

Both saccharogenic method and the Wohlgemuth technique have been applied to urine.

#### The Wohlgemuth Method

This method is essentially that of Harrison (1947).

**Reagents.** 1. Stock starch solution. This contains 2 grams of starch and 10 grams of sodium chloride per 100 ml. of solution. Make 2 grams of starch into a paste with a few ml. of water and pour into about 60 ml. of boiling water, washing in the starch with portions of a few ml. of water. Add 10 grams of sodium chloride and dissolve. Cool, transfer to a 100 ml. volumetric flask and make up to the mark.

2. Starch solution, 0·1 per cent., for use. Dilute 5 ml. of the stock solution to 100 ml. with water. This solution should not be kept for more than a day or two.

3. Approximately N/50 iodine solution. Prepare by diluting a small amount of N/10 iodine 1 in 5.

**Technique.** Adjust the *p*H of the urine so as to be just acid to litmus using strong sodium hydroxide solution or concentrated hydrochloric acid and an appreciable amount of urine so that there is negligible alteration in volume. Put up a series of tubes as follows, taking care when pipetting not to allow any contamination with saliva (salivary amylase):

| Tube | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Diastatic index | 2 | 4 | 6·7 | 10 | 14·3 |
| Ml. urine | 1·0 | 0·5 | 0·3 | 0·2 | 0·14 |
| Ml. water | 0·0 | 0·5 | 0·7 | 0·8 | 0·86 |

| Tube | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|
| Diastatic index | 20 | 28·6 | 40 | 50 | 67 | 100 | 133 | 167 |
| Ml. of urine (1 in 10) | 1·0 | 0·7 | 0·5 | 0·4 | 0·3 | 0·2 | 0·15 | 0·12 |
| Ml. of water | 0·0 | 0·3 | 0·5 | 0·6 | 0·7 | 0·8 | 0·85 | 0·88 |

Continue similarly with a 1 in 100 dilution of urine if necessary. Add 2 ml. of the 0·1 per cent. starch solution to each tube and place the tubes in a water bath at 37° C. for thirty minutes. On removing from the bath place the tubes immediately in cold water for a minute or two. Then set them up in a rack and add the iodine solution slowly, drop by drop, to each tube and observe carefully whether any blue colour is produced. The smallest volume

of urine which does not give any blue colour is noted. This tube may show a reddish colour due to the presence of erythrodextrin.

**Calculation.** The diastatic index is defined as the number of ml. of 0·1 per cent. starch digested by 1 ml. of urine at 37° C. in thirty minutes. This is given by the formula

$$\text{Diastatic Index} = \frac{2}{\text{Smallest volume of urine not giving a blue colour.}}$$

Thus if tube 11 is the first to show a blue colour with iodine, the least amount of urine just able to digest 2 ml. of 0·1 per cent. starch is that in tube 10, that is 0·3 ml. of 1 in 10 urine or 0·03 ml. of urine. Hence

$$\text{Diastatic Index} = \frac{2}{0{\cdot}03} = \frac{200}{3} = 67 \text{ units.}$$

The dilution of the urine is continued until a point is reached at which digestion is incomplete. With high values of the diastatic index it may be necessary to use 1 in 100 urine and even 1 in 1,000 occasionally.

**Note.** The estimation is carried out on a twenty-four-hour specimen of urine which may be preserved with a little toluene, except in the case of patients suffering from acute abdominal conditions, when the test is urgent and is done on a single specimen of urine.

**Normal range.** The urine amylase or diastase normally corresponds to a diastatic index from 5 to 20 units. This applies to twenty-four-hour specimens. If a single specimen of urine is used wider normal variations are found and may reach to about 50 units with more concentrated urines.

### SERUM AND URINE AMYLASE INTERPRETATION

Variations in urinary amylase reflect alterations in serum amylase so long as the kidneys are functioning normally. In renal disease serum amylase may be increased and urine amylase be low.

Considerable increases in both are seen in acute pancreatitis. In Somogyi units the serum amylase may exceed 1,000 units per 100 ml. whilst the urinary diastatic index in the above Wohlgemuth units may exceed 1,000 units. The increase reflects the absorption of enzyme released in the interstitial tissue of the pancreas, in the early stages of the disease. There is often a rapid fall to normal levels, which may be reached again within a few days. For example, the following figures were obtained by the author on successive days in a case of acute pancreatitis, 1,100, 465, 165, units per 100 ml. This does not necessarily indicate that the attack is subsiding, since it may be due to destruction of active secreting tissue. So it is clearly important to do the estimation as soon after the onset as possible or the rise may be missed. Urinary amylase also falls rapidly but the return to normal may take a little longer than with the serum amylase.

In chronic disease of the pancreas, whether due to neoplasm or to chronic pancreatitis such marked increases are not seen. Either duct obstruction is more gradual or progressive destruction of secreting tissue largely counteracts its effect. Increases when they occur are usually small and do not exceed 100 to 200 units per 100 ml. of serum, with similar values for urinary amylase.

There are a few instances of other abdominal diseases such as intestinal obstruction, acute peritonitis, perforated peptic ulcer, in which increases in serum amylase have been observed. These do not exceed 500 units per 100 ml., the majority rising to between 200 and 300 units. These usually develop five to ten days after the onset of the symptoms, and appear to be due to increased pressure on the pancreatic duct. (Raffensperger, 1950). An increase may occur in mumps, and in calculous disease of the salivary glands.

It is seen that results over 500 units per 100 ml. for serum amylase are diagnostic of acute pancreatitis.

Low values of both serum and urinary amylase may be present in liver disease.

### Determination of Serum Lipase

Several methods have been published for the determination of serum lipase, but none is entirely satisfactory. It is clear that there are two distinct types of enzyme which are able to hydrolyze esters of fatty acids. One, esterase, which occurs in liver, kidney, blood serum, and pancreas, acts particularly on esters of short chain fatty acids. The other, lipase, exists almost solely in the pancreas, and is able to hydrolyze esters of long chain fatty acids containing 8 to 18 carbon atoms. Thus as substrate for lipase, esters of long chain fatty acids are to be preferred, but even triolein (olive oil) is hydrolyzed to a small extent by esterases. Esters such as tributyrin are hydrolyzed by both groups of enzymes.

As a consequence there is some lack of specificity in methods suggested for measuring lipase activity. Triolein appears to be the best substrate though the rate of hydrolysis is slower than with the short chain fatty acid esters. Methods using tributyrin measure both types of enzyme, though even so may give useful information. This is also true to some extent with Tween 20 (see p. 276), which, unlike triolein and tributyrin is miscible with water.

**I. Method of Cherry and Crandall** (Cherry and Crandall, 1932; Comfort and Osterberg, 1934; for a later modification see Maclay, 1948).

**Reagents.** 1. Olive oil, 50 per cent. emulsion. Homogenize a mixture of equal parts of olive oil and a 5 per cent. solution of gum acacia containing 0·2 per cent. of sodium benzoate as preservative.

2. Phosphate buffer, *p*H 7. Mix 100 ml. of M/3 disodium hydrogen phosphate (47·3 grams $Na_2HPO_4$ per litre) and 30 ml. of M/3 potassium dihydrogen phosphate (45·3 grams $KH_2PO_4$ per litre).

3. Ethyl alcohol. Ninety-five per cent. (v/v).

4. Sodium hydroxide. N/20.

5. Phenolphthalein, 1 per cent. solution in alcohol.

**Technique.** Pipette 3 ml. of distilled water into each of two test tubes and add 1 ml. of serum to each. Place one in a water bath at 100° C. for five minutes to inactivate the lipase, and then cool. To both tubes add 0·5 ml. of the buffer solution and 2 ml. of the 50 per cent. olive oil emulsion. Shake well and incubate at 37° C. for

twenty-four hours. Then, after adding 3 ml. of 95 per cent. alcohol and 2 drops of phenolphthalein, titrate each with N/20 sodium hydroxide to a similar pink colour.

**Calculation.** Express the result in terms of ml. N/20 sodium hydroxide required to titrate the fatty acid liberated. Then
Units lipase activity per ml. of serum.

= Ml. titration of the unknown — ml. titration of control.

Normal sera are stated by Comfort not to exceed 1·5 units. Other workers have given slightly different figures, and 1·0 unit has been considered the upper normal limit. There can be no doubt that above 2·0 units is pathological, while figures below 1·0 are normal. Values between 1·0 and 2·0 are somewhat equivocal. The author would support the lower value of 1·0 units as the upper limit of normal. It has been held there is no lipase in normal sera.

**Note.** It is recommended that for values above 4 units the serum should be diluted with water or a shorter time of digestion used, so as to bring the difference between the titrations to about 3 ml. Make the necessary adjustment to the calculation. This procedure extends considerably the range found in disease, since with increasing amounts of lipase the enzyme activity is not proportional to time, but tapers off markedly (see Jackson *et al.*, 1952). Values up to 20–30 units may be obtained in acute pancreatitis. However, almost all the published figures appear to have been obtained using 1 ml. of undiluted serum and twenty-four hours incubation.

### II. Tributyrin Method of Goldstein and Roe (1942–43)

**Reagents.** 1. Tributyrin.

2. Taurocholate emulsifying agent. Add 50 grams of sodium taurocholate to 50 ml. of water and autoclave at 15 lb. pressure for two hours. Cool. Add 500 ml. of glycerol and heat the mixture on a water bath until a clear solution is obtained. Stopper and keep in the refrigerator. Before use, warm to 60° to 70° C. to facilitate pipetting and measuring.

3. Barbitone buffer. Dissolve 5 grams of sodium barbitone in water and make up to 1 litre.

4. Calcium acetate solution. Dissolve 20 grams of $Ca(CH_3COO)_2$, $H_2O$ in water and make up to 1 litre.

5. Alcohol-ether mixture. Mix 9 parts of alcohol with 1 part of ether.

6. Alcoholic potassium hydroxide, N/10 solution.

7. Indicator. Phenolphthalein, 1 gm. per 100 ml. of 95 per cent. alcohol.

**Technique.** Pipette 0·25 ml. of the substrate, tributyrin, into a 6 × 1 inch test tube and add 5 ml. of the taurocholate-glycerol mixture. Shake well and place in boiling water for five minutes. Shake thoroughly to emulsify the tributyrin and add 10 ml. of the barbitone buffer and 10 ml. of the calcium acetate. Add 1 ml. of serum and incubate for one hour at 37° C. Prepare another tube in the same way and incubate similarly for one hour, but without

addition of serum. At the end of an hour, remove, cool, and add 1 ml. of serum to the second tube. Pour each immediately into 250 ml. flasks containing 100 ml. of the alcohol-ether mixture, washing in with a little more of this mixture. Add 1 ml. of 1 per cent. phenolphthalein and titrate both to the same pink colour with N/10 alcoholic potassium hydroxide.

**Calculation.** The unit used represents the amount of enzyme activity per 100 ml. of serum which liberates fatty acids equivalent to 1 ml. N/10 potassium hydroxide. Hence

Units per 100 ml. serum = (titration of unknown − titration of blank) × 100.

The normal range was given by the authors as 35 to 110.

**Notes.** 1. The substrate can be prepared more conveniently by the use of an homogenizer, which gives a better and more stable emulsion.

2. Tributyrin is hydrolyzed by esterases, but it has been shown that taurocholate diminishes the activity of esterases and enhances that of lipases.

3. It is recommended that blood should be collected in the morning about fifteen hours after food was last taken by the patient.

4. *Simplified Method* (Goldstein *et al.*, 1948). Pipette 0·75 ml. of tributyrin, 30 ml. of barbitone buffer, 30 ml. of calcium acetate solution, and pass through a hand homogenizer three times. Measure 20 ml. into two 6 × 1 inch test tubes, using one as control. Place in a water bath at 37° C., add 1 ml. serum to one tube. Mix. An hour later add 1 ml. of serum to the control and add the contents of each tube to 100 ml. of alcohol ether mixture and as before titrate with N/20 potassium hydroxide using phenolphthalein. The same unit is used.

*Other Methods.* Archibald (1946) used Tween 20, a polyoxyalkylene derivative of sorbitan monolaurate, as substrate. Since this is soluble in water it is not subject to the objections which can be brought against the above methods, that the emulsions tend to break up during the digestion and do so to a variable extent. Seligman and Nachlas (1950) have used β-naphthyl laurate as substrate. β-naphthol is liberated and then estimated colorimetrically after coupling with tetrazotized diorthoanisidine.

Up to the present the method of Cherry and Crandall has been almost exclusively used so that almost all the figures for serum lipase given in the literature are in their units.

INTERPRETATION

As with serum amylase, serum lipase is increased in acute pancreatitis. Values up to 10 Cherry Crandall units have been reported. It has been stated that serum lipase falls less quickly than serum amylase, so that whereas the latter may return to normal within two to six days, the lipase may take a few days longer. This, however, is not always the case. Significant increases are not so commonly found in other pancreatic conditions. Lipase has been said to be

more frequently raised than amylase in carcinoma of the pancreas and in carcinoma of the ampulla of Vater. It is increased in about a third of such cases when admitted to hospital. Serum lipase has been reported to show appreciable increases in those abdominal lesions, perforated peptic ulcer, intestinal obstruction, acute peritonitis, which show increases in serum amylase. Raffensperger (1950) obtained values up to 6·2 units.

**Determination of Serum Enzymes after Stimulation of the Pancreas**

Attempts have been made to develop more sensitive tests to reveal the presence of pancreatic disease, by stimulating the pancreas to produce its external secretion and then studying the effect on the level of the serum enzymes. Pancreatic stimulants used have included secretin and pancreozymin prepared from extracts of duodenal mucosa, and the vagal stimulants mecholyl and prostigmine. This type of test can be used in two ways :

1. To show that destruction of pancreatic tissue has occurred. In such cases a maximum stimulus is required so as to give an appreciable increase in serum enzymes in normal cases. Then a failure to produce such an increase would show impaired pancreatic function.

2. To show the presence of some degree of obstruction in the pancreas. The most satisfactory form is that in which the stimulus just fails to produce an increase in normal persons, but which will produce one in cases with pancreatic disease.

The second of these appears to be more promising. When there is a marked reduction in the enzyme secretion of the pancreas, other tests are often abnormal, such as studies of the fæces. Knight, Muether and Sommer (1949) gave 1 mg. of prostigmine methyl sulphate intramuscularly, and estimated serum amylase half hourly for two hours afterwards. No change was found in normal persons. They obtained characteristic alterations in obstruction of the duct but in individual cases the response was not definite enough to be of diagnostic value.

The use of hormonal stimulation appears to be more satisfactory. Lopusinak and Boekus (1950) gave secretin and estimated serum amylase and lipase at 0, 1, 4, and 24 hours. In normal cases no increase was observed, but in cases of pancreatitis with high initial values, and in cases of acute pancreatitis within ten weeks from the onset of the attack, a rise in serum amylase was found. An increase was not often seen in chronic pancreatitis and carcinoma of the pancreas when the fasting levels were normal.

Duncan *et al.*, (1950) have estimated serum amylase following intravenous injection of secretin and pancreozymin and found that whilst no change was obtained in normal cases, significant increases could be obtained in some cases of carcinoma head of pancreas and in the early phases of chronic relapsing pancreatitis. Further work is in progress with the object of working out the most satisfactory dose of stimulant. In this way it is possible that a test may be devised which will give more information than simple estimation of serum enzymes. Limitations of this form of test arise from the fact

that some degree of obstruction must be present and that there must be sufficient functioning tissue. Should there be extensive destruction of secreting cells, no increase will be obtained even when there are widespread pathological changes.

## EXAMINATION OF THE FÆCES

While quantitative analysis gives the most reliable indication of the presence of abnormal fat and nitrogen digestion, microscopical examination may give useful information.

### Microscopical Examination of Fæces

Full microscopical examination includes the following :

1. Crystals. Phosphates, oxalates, calcium carbonate, calcium sulphate, cholesterol, Charcot Leyden crystals.
2. Food residues. Undigested and unabsorbed foods. These include fats, muscle fibres, starch granules, indigestible constituents, such as vegetable cell residues, cellulose fibres and connective tissue.
3. Cells, etc. Blood cells, epithelial cells, amœbæ, cysts, parasites and ova.
4. Foreign bodies, for example hairs.

Only the first two groups will be discussed. For cells, ova, and parasites see Cammidge (1914) and Stitt (1948).

For ordinary investigation mix a little of the fresh fæces with saline on a slide, put on a cover glass, and examine first with the 2/3 and then in greater detail with the 1/6 objective. A second slide is often put up at the same time, the fæces being mixed with a little Lugol's iodine. Additional information can be obtained by staining with this and other stains and by adding solvents to the material on the slide.

**Crystals.** *Triple phosphate* crystals are normal constituents of fæces and occur as " coffin lid " shaped crystals and in less regular forms. *Calcium phosphate* crystals are usually seen as sheaves. *Calcium oxalate* crystals, which are similar in shape to those found in urine deposits, occur most frequently when fruits and vegetables rich in oxalates are eaten. Crystals of *calcium carbonate* and *calcium sulphate* occur only occasionally. The solubilities of these inorganic crystals are described under urine deposits where an account of their more characteristic shapes is also given.

*Cholesterol* crystals are sometimes seen in their most typical form (see Fig. 28), but they may be much less characteristic so that they can only be definitely recognized chemically or by their solubility. They dissolve easily in chloroform and in hot alcohol but not in cold alcohol.

*Charcot Leyden crystals* are seen as almost needle shaped octahedra (Fig. 48). They are sometimes found in large numbers in the fæces of patients with worms and embedded in mucus in mucous colitis.

FIG. 48. Charcot Leyden Crystals.

They are, however, present in other diseases and are not characteristic of any condition.

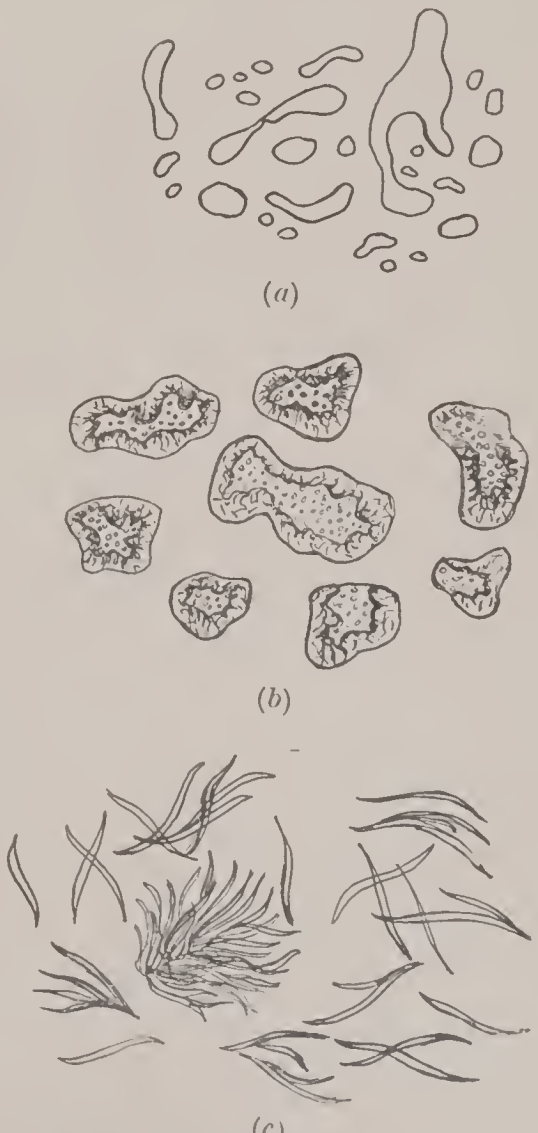
Drugs may sometimes be present either crystalline or amorphous.

**Fats.** The different types of fat which are present in fæces (see p. 282) may be distinguished as follows :

*Neutral fats* are seen as oily globules, (Fig. 49 (*a*)) usually colourless, but if they have a higher melting point they appear as flakes which may be stained with bile pigments. They dissolve readily in cold alcohol. If oily globules are present it is advisable to verify that the patient has not been taking liquid paraffin. Sudan III in concentrated alcoholic solution stains neutral fats orange to red.

*Fatty Acids* appear as colourless needle-shaped crystals, (Fig. 49 (*c*)) usually in groups, but they may occasionally form flakes. They melt on heating. Sudan III stains the flakes but not the crystals.

*Soaps* form masses of needle-shaped crystals and are also seen as amorphous oval-shaped flakes. These often have rolled-over edges and and are termed soapy plaques (Fig. 49 (*b*)). They do



(*a*)

(*b*)

(*c*)

FIG. 49. Microscopic Examination of Fæces. Types of fat present.

not dissolve in ether or alcohol, which distinguishes them from fats and fatty acids. They stain green on the slide when a few drops of saturated copper nitrate solution are added, whereas fatty acid crystals are not affected. Furthermore they do not melt on heating. They do not stain with Sudan III. In some fæces an excess of soaps may be present, yet are not seen on microscopic examination. The following procedure has been suggested for revealing this. Mix the fæces with 70 per cent. Sudan III in alcohol and acetone (equal volumes). This stains fatty acids and neutral fat. Take another specimen of the fæces, acidify with 30 per cent. acetic acid and boil for a few minutes. Cool, and stain again. The acid hydrolyzes the soaps so that an increased amount of fatty acids then shows that the fæces contained an increased quantity of soaps.

In normal fæces, only soaps appearing as a few plaques are observed. Fat droplets are not seen on ordinary diets and their

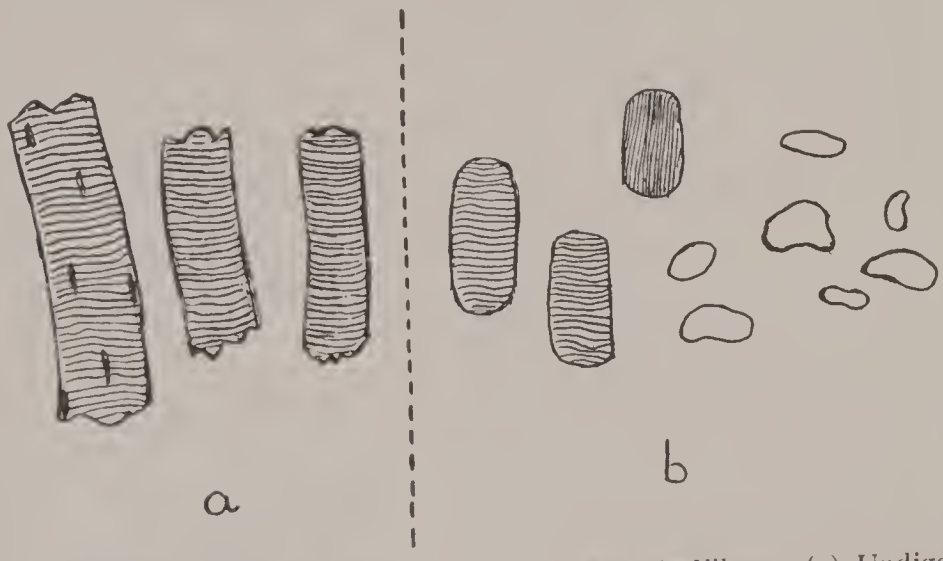



Fig. 50. Microscopic Examination of Fæces. Muscle Fibres. (*a*) Undigested. (*b*) Partly digested.

presence suggests that there is some pancreatic deficiency. Appreciable numbers of fatty acid crystals and excessive amounts of soaps point to defective absorption. The significance of increases in the different types of fat is discussed more fully later (see p. 289).

**Muscle Fibres.** A typical muscle fibre is shown in Fig. 50. It has irregular ends, visible nuclei and crosswise striations. When acted on by the digestive juices the nuclei disappear first, then the ends become smooth and rounded. Subsequently the crosswise striations disappear leaving much fainter longitudinal ones which also finally disappear. The course of digestion is shown from left to right in Fig. 49. Some vegetable fibres found in fæces may be rather similar in shape to muscle fibres. However, they usually have straighter, more well defined ends, and the striations are discontinuous (see Fig. 51). Furthermore, the muscle fibre striations stain with Heidenhain's iron hæmatoxylin whereas the markings on vegetable fibres do not.

A few partially digested muscle fibres are seen in stools from normal people taking an ordinary diet containing meat. These do not average more than one to two per field and frequently there is less than one. The occasional fibres seen in normal stools have rounded ends, sometimes with striations, often without. In pancreatic disease there may not only be a marked increase in the number of fibres seen, but fibres with irregular ends either with or without nuclei may be observed (Fig. 50 (*a*)). The term *creatorrhœa* is used when such fibres are seen in the fæces. Whilst such undigested muscle fibres point to the existence of impaired pancreatic function, the converse is not the case. Protein-splitting enzymes may be derived from

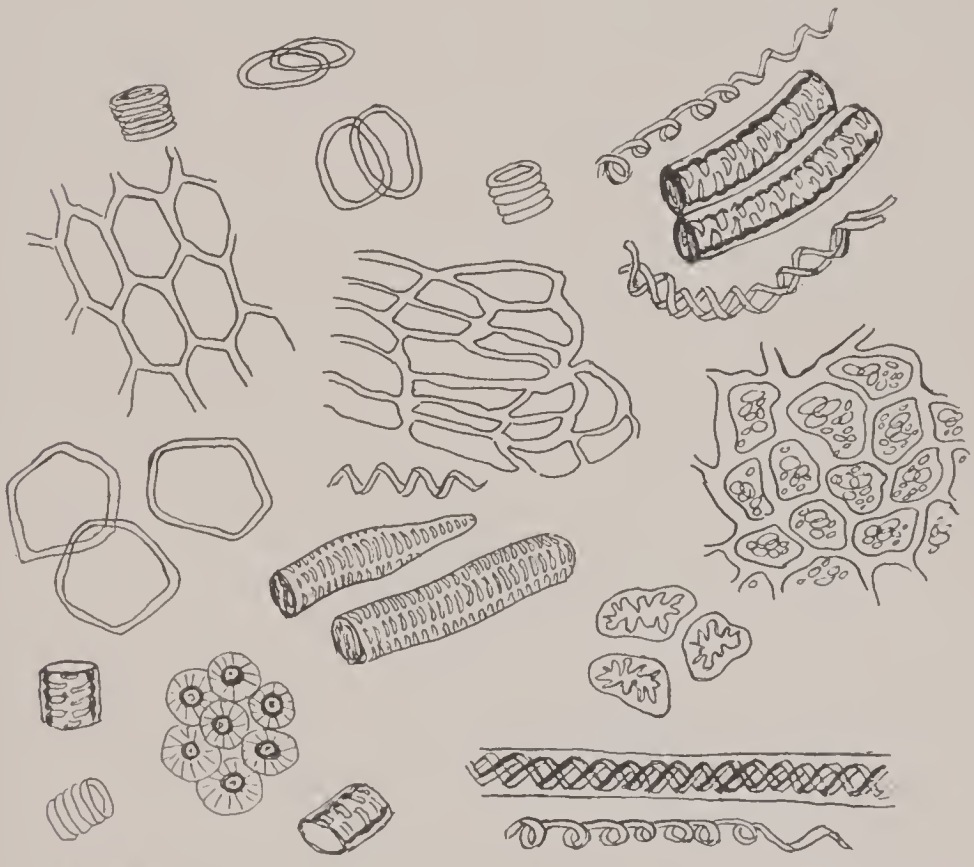


Fig. 51. Microscopic Examination of Fæces. Vegetable Fibres.

other sources, including bacteria. Consequently extensive pancreatic disease may be present even though no such undigested muscle fibres are seen in the fæces.

**Starch Granules.** In order to identify these, Lugol's iodine must be used. This stains starch granules blue or purplish blue. The most important cause of an increased quantity of starch in the stools appears to be an increased rate of passage through the intestine. This also appears to be the main cause of the increase in the number of starch granules seen in all types of steatorrhœa. The amount of starch seen is also influenced by the toughness of the cellulose envelopes which is greater in uncooked than in cooked starch and may be diminished by thorough mastication. Under ordinary conditions

starch granules are uncommon in the stools of healthy adults but are more often seen in the fæces of infants.

Examination for starch granules is of little value in disease. Even in pancreatic disease with marked reduction in the secretion of amylase, salivary amylase and intestinal bacteria are able to break down the starch so that granules are not more frequently seen than in other types of steatorrhœa.

**Vegetable Fibres and Cells.** A wide variety of vegetable cells and fibres from foods eaten is seen when fæces are examined microscopically. Examples of these are shown in Fig. 51. Technicians should become familiar with their appearance. As far as chemical examination is concerned some fibres may be mistaken for muscle fibres whilst others may be thought to be worms. For more information on these topics, see Stitt and Cammidge, already referred to.

### Determination of Fæcal Nitrogen

In pancreatic disease with diminished secretion of protein-splitting enzymes the fæcal nitrogen may be increased. In health on a mixed diet the amount of nitrogen in the fæces is largely independent of the amount of nitrogen in the diet but is increased when diets with a high residue are taken. On a diet containing 101·6 grams of fat and 117 grams of protein (18·8 grams of nitrogen) Dornberger *et al.*, (1948) found an average daily excretion in the fæces of 1·7 gram of nitrogen the range being from 0·8 to 2·5, corresponding to 8·9 per cent. of the ingested nitrogen, range 4·3 to 13·2 per cent. They also investigated ten cases of chronic pancreatitis in which diabetes and calcification could not be demonstrated, and a further ten cases in which both these were present together with steatorrhœa in seven of them. In the first group there was no significant change in the nitrogen excretion but in the second group the range was from 1·7 to 5·6 grams, corresponding to 9·0 to 29·8 per cent. of the ingested nitrogen, excreted daily. These workers also studied three cases after resection of the head of the pancreas and ligation of the pancreatic duct, and two patients after total pancreatectomy. The percentage of nitrogen lost in the fæces by these patients ranged from 30 to 55 per cent. of that taken in the diet. Even higher figures have been reported by other workers.

Fæcal nitrogen is determined by the Kjeldahl method. Homogenize the fæces and take a suitable aliquot.

## Fat in Fæces. Steatorrhœa

The fat present in the fæces is in three forms.

1. Neutral fat, the triglycerides of the fatty acids. This is the form in which fat is mainly present in the food.
2. Free fatty acids, which result from the splitting of the neutral fat by lipases in the intestine.
3. Combined fatty acids, or soaps, the alkali and alkaline earth salts (sodium, potassium, calcium) of the fatty acids. These are formed in the intestine from the fatty acids liberated there.

Neutral fat is thus fat which has not been digested and is referred to as *unsplit fat*, whilst free fatty acids and soaps represent fatty acids formed by digestive action but which have not been absorbed and so are termed *split fat*. It should be noted that neutral fat and free fatty acids are soluble in ether whereas soaps are not, and have to be hydrolyzed by acid, before they can be extracted with ether. Neutral fat, on the other hand, is hydrolyzed by alkalies.

### Determination of the Fat Content of Fæces

Methods for estimating the amount of fat have used both the wet and dried fæces. It has been held by those who used wet fæces that the process of drying may drive off some of the more volatile fats, and that there may be some hydrolysis of neutral fat thus giving rise to misleading information about the percentage splitting of the fats. However, many workers have continued to hold the view that significant errors are not introduced by careful drying. A simple method for dried fæces is that of Cammidge using modified milk fat tubes (Cammidge, 1914).

#### I. Cammidge's Method using Dried Fæces

**Reagents.** 1. Hydrochloric acid, 30 per cent, v/v. Dilute 30 ml. of the concentrated acid to 100 ml. with water.

2. Ether.

3. Alcoholic sodium hydroxide, N/10 solution.

4. Phenolphthalein as indicator.

**Technique.** Most specimens of fæces can be dried satisfactorily at about 50 to 60° C. in a hot air oven, although this may take two or three days. Drying may be carried out on a water bath. In any case a complete stool should be used since fat distribution may be irregular. Appreciable errors may be introduced if samples of a few grams only are dried. Place the specimen in an evaporating dish and break up at intervals to expose as large a surface as possible. With fæces containing a large amount of fat, drying is more difficult. Addition of alcohol has been used since this reduces the boiling point and speeds up the evaporation. Dry the fæces to constant weight, and when dry, powder well.

Weigh out two 1-gram portions, and place one each into two of the tubes shown in Fig. 52. Run 10 ml. of 30 per cent. hydrochloric acid into one and 10 ml. of water into the other. Place the tube containing the acid, loosely corked, into a bath of boiling water and heat for about fifteen minutes, shaking occasionally. This hydrolyzes the soaps. Cool thoroughly.

Fig. 52. Cammidge Tubes for Fat in Fæces.

To each tube add ether to the 50 ml. mark, cork firmly, and mix well by inverting frequently. Do this several times for two or three minutes at intervals and finally allow the solid part to settle out completely. Weigh two small beakers of about 50 ml. capacity and pipette 20 ml. of the clear ether extract from each of the tubes and run into the beakers. Allow the ether to evaporate, either spontaneously, or carefully in a hot air oven at about 35° C., and heat finally to about 60° to 70° C. to constant weight. Weigh the beakers to obtain the weight of fat in them.

Dissolve the fat in the beaker from the tube containing water, in ether, wash into a suitable flask, and titrate the free fatty acids present with N/10 alcoholic sodium hydroxide using phenolphthalein as indicator. To convert the titration into grams of fatty acids multiply the number of ml. used by 0·0277.

**Calculation.** We thus obtain :

1. The weight of fat from the acid tube = the weight of total fat in 0·5 gram of dried fæces.
2. The weight of fat from the water containing tube = the weight of free fatty acids + neutral fat in 0·5 gram dried fæces.
3. The number of ml. N/10 sodium hydroxide required to titrate the fat in the beaker from the water containing tube × 0·0277 = the weight of free fatty acids in grams in 0·5 gram of dried fæces.

From these we get the following :

(1) × 200 = *total fat*, as percentage weight of the dried fæces.
(3) × 200 = *free fatty acids* as percentage weight of the dried fæces.
[(2) − (3)] × 200 = *neutral fat* as percentage weight of dried fæces.
[(1) − (2)] × 200 = *soaps* (*combined fatty acids*) as percentage weight of the dried fæces.

$$\text{Then, } \textit{Split fat} = \frac{\text{Soaps + Free fatty acids}}{\text{Total fat}} \times 100, \text{ as percentage of the total fat.}$$

$$\textit{Unsplit fat} = \frac{\text{Neutral fat}}{\text{Total fat}} \times 100, \text{ as percentage of the total fat.}$$

### II. Method of Holt, Courtney, and Fales (1919) using Dried Fæces

This method, in many respects similar to the Cammidge method, uses a Roehrig tube (Fig. 53) and extracts the fat with different solvents.

**Reagents.** 1. Hydrochloric acid, 30 per cent. v/v as before.
2. Alcohol, either absolute or 95 per cent.
3. Ether.
4. Petroleum ether (b. pt. 40° to 60° C.).
5. Benzene.
6. N/10 alcoholic sodium hydroxide.
7. One per cent. phenolphthalein in 50 per cent. alcohol.

**Technique.** Dry the fæces as described above. Weigh out 1 gram of dried fæces and heat with 10 ml. of 30 per cent. hydrochloric acid on a water bath, mixing well. Do not evaporate to dryness. Cool. Transfer to a Roehrig tube, washing in with distilled water up to mark "½." Then add alcohol to mark "0" and ether to 25 ml.

Stopper firmly and shake well for about a minute, add petroleum ether to the 50 ml. mark and again shake well. Stand for the layers to separate. This separation can be speeded up by rotating the tube while held upright between the palms of the hands. Then run off the ether through a fat-free filter paper into a weighed light flask (about 200 ml.). Repeat the extraction twice, each time using 20 ml. of ether and 20 ml. of petroleum ether. Wash the tap and filter paper with mixed ether and petrol ether. Evaporate off the solvents, fitting the flask with a condenser, so collecting the mixed ether distillate which can be used for the third of the extractions. Allow the flask to stand in a desiccator overnight, clean the outside and weigh. The difference in weight gives the amount of total fat in the dried fæces taken.

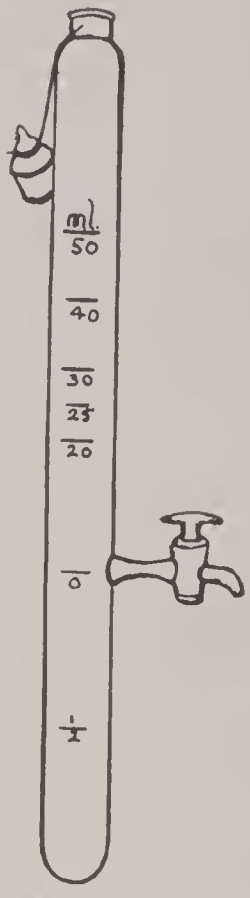


FIG. 53. Roehrig Tube for Fat in Fæces.

Dissolve the fat by heating on a water bath with about 50 ml. of benzene and titrate hot with N/10 alcoholic sodium hydroxide using 0·5 ml. of the phenolphthalein as indicator. This titrates the free fatty acids originally present and those liberated from the hydrolysis of the soaps present, that is, the titration measures the split fat.

**Calculation.** Weight of fat extracted × 100 = percentage total fat by weight of dried fæces.

Ml. titration × 0·0277 × 100 gives weight of split fat in 100 grams of fæces.

Split fat and unsplit fat in terms of percentage of total fat can be calculated as shown before.

**Notes.** 1. It has been claimed that this gives a more complete extraction of fats than does the Cammidge method. The author has not found a significant difference between the two methods.

2. If desired, soaps may be estimated as in the Cammidge method by using 10 ml. of water instead of acid, extracting as above and titrating as in that method. It is not usual to do so but only to report total fat and the percentage splitting.

### III. Fowweather's Simplified Method using Wet Fæces (Fowweather and Anderson, 1946)

Fowweather (1926) described a method using wet fæces based on the method of Saxon (1914). Subsequently he developed a simplified method also using wet fæces.

First grind the specimen thoroughly in a mortar taking particular care to ensure that all coarse particles are reduced so that the specimen has a uniform consistency. Take a porcelain dish and a glass rod flattened at one end, and weigh them. Transfer 8 to 10 grams of fæces to this and again weigh the dish and rod. Then withdraw 2

to 3 grams of the fæces on the flattened end of the rod, and place this in a second mortar. To obtain the weight of fæces removed, weigh the remaining fæces, dish, and rod. Then place the latter in a steam oven and dry to constant weight and so determine the water content of the fæces. Mix the weighed portion of the fæces in the mortar thoroughly with 2 ml. of concentrated hydrochloric acid, add 20 grams of plaster of Paris, grind well together, and leave for thirty minutes. Regrind and extract with dry light petroleum ether in a Soxhlet for three hours. Remove the solvent from the extract on a water bath, and direct a current of air on to the surface of the extracted fat for about two minutes to help to remove any hydrochloric acid still present. Place the flask containing the fat in a steam oven for about thirty minutes. Weigh. This gives the total fat in the specimen. Dissolve the fat in about 30 ml. of benzene and titrate with N/10 alcoholic sodium hydroxide as before. This gives the split fat. The calculation is completed as described previously.

### IV. Method of van de Kamer, Huinink and Weyers (1947) using Wet Fæces

This is a convenient and rapid method and is particularly suited for use in connection with fat balance tests (see later, p. 290). Results are expressed in terms of fatty acids. If a wet method is to be used this is recommended.

**Reagents.** 1. Ethyl alcohol, 96 per cent. containing 0·4 per cent. amyl alcohol.

2. Ethyl alcohol, 96 per cent., neutral to thymol blue.

3. Potassium hydroxide, 33 per cent. solution.

4. Hydrochloric acid, 25 per cent., S.G. 1·13.

5. Hydrochloric acid, 2·5 per cent., S.G. 1·013, to which 250 grams of sodium chloride are added per litre.

6. Petroleum ether (40° to 60° C.). This should leave no titratable residue when evaporated.

7. N/10 sodium hydroxide.

8. N/10 isobutyl alcoholic potassium hydroxide. Van de Kamer *et al.* prepare this as follows. Boil isobutyl alcohol for three hours with sodium hydroxide, 20 grams per litre. Distil, collecting the fraction between 105° and 108° C. To 1 litre of this add 3 grams of concentrated 50 per cent. potassium hydroxide solution (prepared by dissolving the solid hydroxide in an equal amount of water) diluted with 20 ml. of methyl alcohol. Standardize against N/10 hydrochloric acid with thymol blue as indicator.

9. Two per cent. thymol blue in 50 per cent. ethyl alcohol.

10. N/10 hydrochloric acid.

**Technique.** (*a*) For *Total Fat.* Weigh approximately 5 grams of the fæces into a 150 ml. flask. Add 10 ml. of 33 per cent. potassium hydroxide and 40 ml. of 96 per cent. ethyl alcohol containing 0·4 per cent. amyl alcohol and boil under a reflux condenser for twenty minutes. Cool thoroughly, add 17 ml. of 25 per cent. hydrochloric acid from a measuring cylinder and cool again. Add 50 ml. of petrol ether, close the flask with a rubber stopper and shake vigorously for a

minute. Allow the petrol ether layer to separate completely and pipette off 25 ml. into a small conical flask. A pressure pipette as shown in Fig. 54 may be used. Add a piece of filter paper to prevent

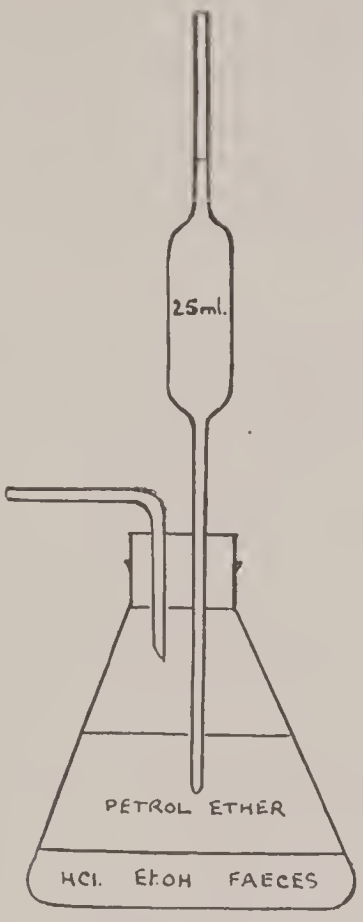



FIG. 54. Pressure Pipette. (*Dr. J. H. Van de Kamer.*)

irregular boiling and evaporate the petrol ether. Then add 10 ml. of 96 per cent. alcohol neutral to thymol blue and titrate with N/10 sodium hydroxide using a microburette and thymol blue as indicator. Alternatively the fatty acids can be titrated directly in the petrol ether layer using N/10 isobutyl alcoholic potassium hydroxide.

**Calculation.** Van de Kamer uses an average molecular weight of 284 for the fatty acids.

Grams fatty acids per 100 grams fæces

$$= \frac{n \times 284 \times 1{\cdot}04 \times 2 \times 100}{10{,}000 \times W} = \frac{5{\cdot}91\ n}{W}.$$

$n$ = ml. N/10 alkali required for the titration.
$W$ = grams of fæces taken for the analysis.

The factor 1·04 was introduced to allow for a 1 per cent. increase in volume of the petrol ether when shaken with alcoholic hydrochloric acid and for 3 per cent. of the fatty acids remaining in the acid-alcohol layer.

(*b*) *Determination of Fatty Acids* (*Free and Combined as Soaps*) *and Neutral Fat Separately.* Weigh approximately 5 grams of fæces in a

cylindrical tube. Add 22 ml. of the 2·5 per cent. hydrochloric acid containing sodium chloride and a little pumice. Boil for one minute under a reflux condenser. Cool thoroughly. Add 40 ml. of 96 per cent. ethyl alcohol containing 0·4 per cent. amyl alcohol and 50 ml. of petrol ether. Close the tube and shake vigorously for a minute.

Allow to separate. Separation can be accelerated by rotating the tube between the hands. Pipette off 25 ml. of the petroleum ether layer into a 100 ml. conical flask as above, add a small piece of filter paper, evaporate off the petrol ether, add 2 ml. neutral ethyl alcohol and titrate the *fatty acids corresponding to the split fat* with N/10 isobutyl alcoholic potassium hydroxide as above in a microburette.

After noting the amount required to neutralize to thymol blue add 10 ml. isobutyl alcoholic potash and reflux gently for fifteen minutes. Add 10 ml. neutral ethyl alcohol and titrate the excess alkali with N/10 hydrochloric acid, until the blue colour turns yellow. From this the amount of *neutral fat*, corresponding to the unsplit fat, can be calculated.

**Calculation.** A molecular weight of 284 is used for the fatty acids and 297 for fat.

Grams fatty acids (split fat) per 100 grams fæces

$$= \frac{5{\cdot}91\ n}{W} \text{ as before.}$$

Grams neutral fat (unsplit fat) per 100 grams fæces

$$= \frac{(B - N)\ 297 \times 1{\cdot}01 \times 2 \times 100}{10{,}000\ W} = \frac{6{\cdot}0\ (B - N)}{W}.$$

B = ml. N/10 hydrochloric acid required to titrate 10 ml. isobutyl alcoholic potassium hydroxide.

N = ml. N/10 hydrochloric acid required for the titration of the test.

For neutral fat a factor of 1·01 is used since the only correction required is for the increase in volume of the petrol ether layer.

**Note.** The water content of the fæces has to be determined if the above figures are to be converted into grams per cent. of the dried fæces.

**Presence of Liquid Paraffin.** If liquid paraffin has been taken and is present in the fæces it will be extracted and will be found to be present in the neutral fat by all the methods except that of van de Kamer, in which it will not be titrated. So liquid paraffin should not have been given for at least three days before the test is to be done. Fæces containing it will remain greasy on drying as do fæces rich in fat. A high fat content with a high percentage of neutral fat is found rather infrequently so the possibility of liquid paraffin being present in such cases should always be borne in mind.

If necessary, liquid paraffin can be tested for by the method of Archer, given by Harrison (1947). Add 5 ml. of concentrated sulphuric acid carefully to the fat obtained after evaporating off the solvents used in its extraction. Heat almost to boiling. Whilst fats

and fatty acids are destroyed, liquid paraffin is unaffected. After cooling thoroughly, pour slowly into about 50 ml. of water in a separating funnel, washing out the vessel with water. Then extract twice with petrol ether using about 20 to 30 ml. Evaporate to dryness and weigh. This will demonstrate the presence of liquid paraffin and allow a correction to be made if desired. It is better to repeat the determination.

**Presence of Barium Salts.** These can usually be recognized quite easily. The fæces are white and heavy. The organic material can be destroyed and the presence of barium shown by the usual tests of inorganic analysis. The specimen is valueless and a further one should be obtained.

INTERPRETATION

It is usual to allow a fat content up to 25 per cent. of the dried weight in the fæces of normal adults. The ratio of split fat to unsplit is at least 3 : 1 so that at least 75 per cent. of the fat present is split fat. In the case of children a fat content up to about 30 per cent. of the dried fæces may occur normally, with up to 33 per cent. of unsplit fat.

With persons on low fat diets giving less than about 10 per cent. of fat in the dried fæces, the ratio of split fat to unsplit fat may be lower than 3 : 1. No significance should be attached to this.

An *increased amount of fat* in the fæces—*steatorrhœa*—may be found in a number of conditions. The presence of such an increase in many cases leads to bulky, shapeless, and frothy stools.

(*a*) In pancreatic disease there is a deficient production of lipase, so that an increase in total fat with an increase of neutral fat and hence of the percentage of unsplit fat might be expected. However this is far from being a consistent finding in pancreatic disease, since pancreatic lipase is not the only lipolytic agent present in the intestine. Some lipase may be produced by the small intestine and bacterial action is also able to bring about some hydrolysis of fats. Cooke *et al.*, (1945) conclude "that the percentage hydrolysis of fat in the fæces has little significance and in particular that it cannot be regarded as a reliable index of normal pancreatic function." It may be agreed that cases of pancreatic insufficiency occur in which there is increase in fæcal fat with normal splitting, and others in which there is even a normal fat content. The most one can say is that whilst there is impaired splitting in some cases of pancreatic disease and this then points strongly to the presence of such disease, normal splitting certainly does not exclude it.

(*b*) In obstructive jaundice there is a deficiency in the amount of bile salts in the intestine, sometimes, if there is associated pancreatic disease, with deficiency of lipase as well. Bile salts act as emulsifiers in fat digestion and absorption, and high percentages of total fat, from 50 to 80 per cent. of the dried weight, may be found when bile is absent from the intestine. A simple bile salt deficiency almost always gives a fat increase with normal splitting.

(*c*) In the group of conditions which includes sprue, coeliac disease

and idiopathic steatorrhœa there is defective fat absorption in the small intestine. Total fat ranges from 30 to 60 per cent. by weight of the dried fæces though there are occasional exceptions. In faulty absorption due to tuberculous disease of the intestine and in chronic dysenteries similar findings have been noted. Normal splitting is found in the great majority of cases.

Conclusions as to the cause of the steatorrhœa drawn from the percentage splitting of the fat are thus seen not to be reliable and some workers have almost ceased to use the determination of the proportion of split and unsplit fat. Criticisms have also been brought against the value of total fat determined on a single specimen of fæces taken under uncontrolled conditions. The amount of fat in the fæces is influenced by the quantity of fat in the diet, and in addition the amount of dry matter in the fæces varies considerably with the type of food taken, and may lead to different percentages of fat not related to variations in fat digestion. Accordingly it has been argued that a closer comparison with the amount of fat taken in the food is desirable. For this purpose fat balance tests are used.

## Fat Balance Tests

The patient is put on a known daily intake of fat which has been variously fixed from 50 to 100 grams. Seventy grams appears to be a suitable amount though 50 grams has often been used. Only the total fat content of the fæces is determined. After the patient has been taking a known amount of fat for two to three days all the fæces passed for a number of days, from three to six usually being adequate, are collected and the total amount of fat excreted during that period is estimated. The daily excretion of fat can then be calculated. The fæces may be dried as they are received, and finally mixed, weighed, sampled, and the fat content of a 1-gram portion determined. Alternatively the method of van de Kamer *et al.*, adapted as follows (personal communication from Professor Frazer, see also Anderson *et al.*, 1952), can be used very conveniently. Each day as the fæces are received, mix thoroughly with 500 ml. (or more if necessary) of water, so as to produce a completely homogeneous suspension. Measure a portion (20 ml.) into a 250 ml. ground glass stoppered conical flask, add 95 ml. of 3 per cent. alcoholic potassium hydroxide solution containing 0·4 per cent. of amyl alcohol, and boil on a steam bath for twenty minutes. Cool. Acidify with 30 ml. of 33 per cent. (v/v) hydrochloric acid. This liberates the whole of the fatty acids present. Add 50 ml. of petroleum ether and shake well for 1 minute. Allow to stand and settle. Take 20 ml. of this petrol ether extract and titrate with N/10 alcoholic sodium hydroxide using thymol blue or phenolphthalein as indicator. One ml. of N/10 NaOH equals 0·0284 gram fatty acid. The total fatty acids present in the specimen of fæces can easily be calculated.

$$\text{Total fatty acids in grams} = \text{Ml. N/10 NaOH required} \times 0{\cdot}0284 \times \frac{50}{\text{volume of petrol ether extract titrated (20 ml.)}}$$

$$\times \frac{\text{total volume of fæcal suspension (500 ml.)}}{\text{volume of fæcal suspension used (20 ml.)}}$$

$$= \text{Ml. N/10 NaOH required} \times 1{\cdot}78$$

for the quantities used above. A litre of suspension may be made sometimes and only 10 ml. of extract may need to be titrated. Adjust the calculation accordingly.

The daily excretion of fat expressed as total fatty acids is thus obtained and the average daily excretion over the selected period can easily be calculated. A useful method of reporting which the author obtained from Professor Frazer, is to determine the daily excretion in terms of the total fatty acids on the fæces passed each day, and then from the third day onwards calculate the average daily excretion for that day and the two previous days. It is usually clear quite soon whether the case is normal or is a definite steatorrhœa, and in borderline cases the test can be carried on for longer periods so that inequalities in the amount of fæces excreted each day have less effect on the result. Little advantage is to be gained over this method by attempting to mark the fæces. The method suggested also makes it possible to evaluate the effect of any treatment which may be given. The results can be conveniently expressed in diagrammatic form.

The normal absorption of fat is at least 90 per cent. of the intake. Frazer and his co-workers, who used 50 grams of fat daily, give $95 \pm 4$ per cent. (Cooke *et al.*, 1945). Similar values are obtained when 70 or 100 grams of fat are given. Thus an absorption of less than 90 per cent. of the intake may be regarded as abnormal, and as showing the presence of steatorrhœa. An absorption between 55 and 90 per cent. is commonly found in steatorrhœa. It is only very occasionally that a lower percentage absorption is obtained.

The fat balance test as thus carried out gives a more reliable indication whether a patient has steatorrhœa than does the determination of the fat content of a random specimen of fæces.

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*CHAPTER XVI*

# CALCIUM, PHOSPHORUS, AND PHOSPHATASES

Since these are all concerned with the calcification of bone, they are often determined together, so it will be convenient to consider them together here. In addition to the part which it plays in the building of bones and teeth, the calcium present in plasma and other extracellular fluid is important for maintaining the correct conditions for normal nerve irritability and muscle contraction. The plasma calcium also plays a part in blood coagulation. The calcium of the bones provides a large reserve which can be drawn on when required. Mobilization of calcium from the bones is controlled by the hormone secreted by the parathyroids.

Phosphorus is more widely distributed than calcium. Besides being present in bones and teeth, unlike calcium it is found in the cells and so is present in the soft tissues. There is phosphorus in the molecules of many important substances in the body, for example, in some proteins, in some lipids, and in many enzymes. The excretion of phosphates affords a means by which the kidneys are able to control the acid-base balance of the body.

## CALCIUM

**Determination of Serum Calcium** (Method of Clark and Collip (1925) ; see also Kramer and Tisdall (1921), and Sendroy (1944) )

The calcium is precipitated as oxalate directly from the serum, and after washing, the precipitate is dissolved in acid and titrated with permanganate.

**Reagents.** 1. Ammonium oxalate, 4 per cent. solution.

2. Ammonia, 2 per cent. (v/v) solution. Dilute 2 ml. of ammonia, S.G. 0·88, to 100 ml. with water.

3. N/100 potassium permanganate solution. Prepare freshly before use by diluting stock N/10 solution.

4. Approximately normal sulphuric acid.

**Technique.** To 2 ml. of serum add 2 ml. of distilled water and 1 ml. of 4 per cent. ammonium oxalate solution. Serum is best used, since although calcium does precipitate from citrated plasma, this occurs more slowly and the results may be unreliable. Oxalated plasma is naturally ruled out. Mix well and allow to stand overnight. If it is necessary to carry out the estimation more quickly, a shorter time of standing may be used, but not less than half an hour. With shaking at frequent intervals complete precipitation can be achieved in that time. Tapered centrifuge tubes are more convenient for this determination. After precipitating the calcium, centrifuge, and remove the supernatant fluid without disturbing the precipitate. With

tapered tubes the supernatant fluid can be poured off. After doing so stand inverted on a filter paper and allow to drain for five minutes. See that the precipitate stays in the tip of the tube. Wipe the mouth of the tube, run 3 ml. of the 2 per cent. ammonia down the inside of the tube, and mix the precipitate with this by means of a fine glass rod which is then kept for later stirrings so that small amounts of precipitate adhering to it are not lost. Centrifuge again, pour off the supernatant fluid, repeat the draining on filter paper, wipe the mouth of the tube and add 2 ml. of approximately normal sulphuric acid. Mix the precipitate with the acid using the glass rod which was used for mixing when washing. Warm by placing in a beaker of almost boiling water to complete solution of the oxalate. Remove and titrate with N/100 permanganate, keeping the mixture at 70° to 75° C., to a faint pink colour which persists for about a minute. Use a 2 ml. microburette graduated to 0·02 ml. As blank, titrate 2 ml. of the sulphuric acid to the same end-point. The difference between these titrations gives the volume of N/100 permanganate required to titrate the calcium oxalate precipitate.

**Calculation.** One ml. of N/100 permanganate is equivalent to 0·2 mg. of calcium. Hence since 2 ml. of serum is used,

Mg. calcium per 100 ml. serum

$$= (\text{Titration of unknown} - \text{titration of blank}) \times 0{\cdot}2 \times \frac{100}{2}$$

$$= (\text{Titration of unknown} - \text{titration of blank}) \times 10.$$

**Notes.** For accurate results the washing must be carefully carried out and standardized. Two factors are involved, removal of the ammonium oxalate solution and possible solution of the precipitate in the wash liquid. A slightly different technique from that given above is as follows. After precipitating the calcium oxalate, and centrifuging, pour off the supernatant fluid without draining and run in 5 ml. of 2 per cent. ammonia. Mix with the precipitate. Centrifuge again, pour off the supernatant fluid and repeat the washing twice, mixing with the precipitate the first time but not the second. Dissolve the precipitate in sulphuric acid and titrate as before.

### Use of Iodometric Titration

An excess of permanganate is used, potassium iodide is added and the iodine liberated from this excess is titrated with N/400 thiosulphate using starch as indicator. A sharp end-point is thus obtained.

**Reagents.** 1. Potassium iodide, 10 per cent. solution made freshly.

2. N/400 sodium thiosulphate. Make freshly for use by diluting 5 ml. of stock N/10 solution to 200 ml.

3. Starch indicator.

**Technique.** Proceed as above up to the point at which the precipitate has been dissolved in sulphuric acid. Then add 2 ml. of N/100 permanganate, stand in hot water until the reaction is complete, cool,

add 1 ml. of potassium iodide solution and titrate the liberated iodine with N/400 thiosulphate.

**Calculation.** Check the equivalence of the solutions by titrating 2 ml. of permanganate, after addition of sulphuric acid and iodide, with the N/400 thiosulphate. Since 1 ml. of N/400 thiosulphate is equivalent to 0·05 mg. of calcium

Mg. calcium per 100 ml. serum

$$= (\text{Titration of standard} - \text{titration of unknown}) \times 0{\cdot}05 \times \frac{100}{2}.$$

$$= (\text{Titration of standard} - \text{titration of unknown}) \times \frac{10}{4}.$$

**de Loureiro and Janz Modification of the Clark Collip Method** (de Loureiro and Janz, 1944)

De Loureiro and Janz have made a study of this estimation and recommend a modified permanganate solution, which can be used either for iodometric or colorimetric estimation of calcium. They claim a more reliable result for the iodometric titration if the reaction is carried out in strong acid solution and in the presence of added manganous sulphate.

**Reagents.** 1. Stock diluent. Dissolve 50 grams of manganous sulphate ($MnSO_4$. $4H_2O$) with moderate heating in 500 to 600 ml. water, cool, add slowly 350 ml. concentrated sulphuric acid, cool, and make up to a litre.

2. N/10 potassium permanganate in the stock diluent. Dissolve 3·5 grams of potassium permanganate in a litre of the stock diluent. Standardize against sodium oxalate and adjust to N/10 with stock diluent. This solution reacts with oxalic acid at room temperature and with potassium iodide in exactly equivalent proportions. The colour of the solution differs somewhat from that of aqueous permanganate solutions. It shows only a very small loss of titre on storage in the refrigerator, a slightly higher one at room temperature. Such change as occurs arises from loss of oxygen and precipitation of manganese dioxide which is black and granular, and sticks to the bottom and walls of the bottle so that the supernatant fluid remains clear and need not be filtered.

3. Working solutions of potassium permanganate. Prepare by diluting the above solution with stock diluent. Never use water since it causes precipitation of manganese dioxide. Use N/100 for the iodometric method.

4. N/100 sodium oxalate solution. Dissolve 0·335 gram of sodium oxalate in 35 per cent. sulphuric acid and make up to 500 ml. with the acid.

5. Nitric acid. Fifty per cent. v/v with water. Dilute 50 ml. of concentrated acid to 100 ml. with water.

6. Potassium iodide, 5 per cent. solution. Prepare freshly for use.

7. N/100 sodium thiosulphate. Prepare by diluting an accurate N/10 solution.

8. Starch indicator. One per cent. starch in saturated sodium chloride solution.

**Technique.** 1. **Iodometric Method.** Prepare and wash the calcium oxalate precipitate as described above. Dissolve in a small drop of nitric acid and add 2 ml. of N/100 potassium permanganate. After thirty minutes add distilled water and wash the contents into a beaker. Add 1 ml. of potassium iodide and titrate with N/100 thiosulphate using a suitable microburette. For the standard, pipette 2 ml. of N/100 permanganate into a flask, add 10–20 ml. of water and 1 ml. of potassium iodide. Titrate with the N/100 thiosulphate. Two ml. should be required.

**Calculation.** One ml. of N/100 thiosulphate = 0·2 mg. calcium. Hence

Mg. calcium per 100 ml. serum

= (Ml. titration of standard − ml. titration of unknown) × 10.

For titration of the oxalate standard, mix 2 ml. of the permanganate and 1 ml. of the oxalate, keep thirty minutes at room temperature, add iodide and titrate with thiosulphate. The difference between the volume of thiosulphate required for the standard and oxalate sample must be exactly 1 ml.

2. **Colorimetric Method.** As before, dissolve the washed calcium oxalate precipitate in a drop of 50 per cent. nitric acid, and add exactly 2 ml. of N/100 potassium permanganate and 3 ml. of stock diluent. Stand for thirty minutes then read in the colorimeter using a green filter or transmission at 520 millimicrons. Let this reading be T. Read also untreated permanganate solution (2 ml. of N/100 solution plus 3 ml. of stock diluent) and let this reading be B.

**Calculation.** The serum calcium is proportional to the difference between the two colorimeter readings, that is

$$\text{serum calcium} = (B - T) \times \text{constant K}.$$

K can be determined as follows, using the standard oxalate solution. Measure out 2 ml. of N/100 permanganate and add 1 ml. of N/100 oxalate and 2 ml. of diluent. Stand for thirty minutes and read in the colorimeter (T). To a further 2 ml. of N/100 permanganate, add 3 ml. of diluent and read in the colorimeter (B). One ml. of oxalate is equivalent to 10 mg. of calcium per 100 ml. serum. Hence

$$10 = (B - T) \times K, \text{ so that K can be found.}$$

A calibration curve can be worked out if desired as follows :

| Mg. calcium per 100 ml. serum | 0 | 2·5 | 5 | 7·5 | 10 | 12·5 | 15 | 17·5 |
|---|---|---|---|---|---|---|---|---|
| Ml. N/100 permanganate | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Ml. N/100 oxalate | 0 | 0·25 | 0·5 | 0·75 | 1·0 | 1·25 | 1·5 | 1·75 |
| Ml. diluent | 3 | 2·75 | 2·5 | 2·25 | 2·0 | 1·75 | 1·5 | 1·25 |

The readings of the oxalate containing specimens can be subtracted from that of the permanganate only, and a straight line curve obtained proportional to the amount of calcium present. It will be

seen that under these conditions it is vitally important that the oxalate solution should be accurate, but that variations in strength of the permanganate will not affect the accuracy of the estimation.

**Note.** This method can be used with smaller quantities of serum, if necessary. It then gives a greater degree of accuracy than is possible with the original Clark Collip method. Whenever possible the full 2 ml. of serum should be used.

*Other Methods.* Roe and Kahn (1929) have used a method which precipitates the calcium from a protein-free filtrate as tricalcium phosphate. This is then washed and determined colorimetrically by the phosphate method given later. Kuttner and Cohen (1927) have described a micro-method based on this principle. This type of method is, however, more troublesome than the above methods, but it is claimed that it can be applied to smaller quantities of serum, an advantage over the Clark Collip method, but not over the colorimetric method described above. In any case, the major errors in determining serum calcium are likely to arise in washing the precipitate. The smaller this is, the greater these errors may be proportionately, so that it is advisable to use 2 ml. serum.

Elliott (1952) has titrated the calcium directly with disodium dihydrogen ethylene diamine tetraacetate using ammonium purpurate as indicator. At *p*H 12 this is specific for calcium. The end-point, however, is not very sharp.

INTERPRETATION

The calcium of the blood is confined to the plasma. Normal serum calcium ranges from 9·0 to 11·5 mg. per 100 ml., the majority of results being near to 10 mg. per 100 ml. The upper limit appears to be slightly higher in very young children. Rather less than half of the calcium is attached to proteins (averaging 4·5 mg. per 100 ml.) and is physiologically inactive. The remaining part (averaging 5·5 mg. per 100 ml.) is active physiologically and it is variations in this fraction which are important pathologically. Serum calcium tends to fall a little in pregnancy. Although usually in the lower part of the normal range, it is occasionally slightly subnormal.

The level of serum calcium may be affected by deficient calcium absorption from the intestine, by alterations in the amount of parathyroid hormone secreted, by changes in the serum inorganic phosphorus, and by alterations in the plasma proteins. In regard to the last of these, it is the physiologically inactive part which is affected; in the other cases it is physiologically active calcium which is altered. In conditions other than those in which there is impaired absorption of calcium and phosphorus, a change in inorganic phosphorus is accompanied by a change in serum calcium in the opposite direction, so that a high inorganic phosphorus is associated with a low serum calcium and vice versa. In this way primary changes in serum inorganic phosphorus can lead to secondary changes in serum calcium.

A reduction in serum calcium affecting the physiologically active

part is associated with tetany. There is a region of latent tetany down to about 8 mg. per 100 ml. Between 7 and 8 mg. per 100 ml. abnormal reflexes can be demonstrated (Trousseau and Chvostek signs), and below 7 mg. per 100 ml. carpopedal spasms may occur. The serum calcium in severe tetany falls to just below 6 mg. per 100 ml. in those cases in which the tetany is due to a reduction in ionized calcium.

**Reduced Serum Calcium.** Some of the lowest values for serum calcium are found in *hypoparathyriodism*. This may arise spontaneously (idiopathic hypoparathyroidism) but occurs more commonly following thyroidectomy carried out on patients with hyperthyroidism when too much parathyroid tissue has been removed. The serum calcium can fall to just below 6 mg. per 100 ml.

Diseases in which calcium absorption is impaired do not as a rule show such low values. In *rickets* the most characteristic abnormality is the low inorganic phosphorus. Serum calcium is most often either just within normal limits or is between 8 and 9 mg. per 100 ml. Only rarely does it fall below 8 mg. per cent. so that symptoms of tetany do not often occur. A low serum calcium is perhaps more frequently observed in adult rickets, osteomalacia. In the *steatorrhœas*—idiopathic steatorrhœa, cœliac disease, sprue—serum calcium is usually between 8 mg. per 100 ml. and normal values. However, a small proportion show lower values and may have active symptoms of tetany.

The high serum inorganic phosphorus of advanced *renal failure* is accompanied by a marked decrease in serum calcium which may be as low as 6 mg. per 100 ml. The acidosis present may counteract the tendency for tetany to occur (see p. 361). Nevertheless, occasionally such patients show typical symptoms of a severe tetany. In those forms of renal disease in which plasma proteins are reduced, serum calcium may be low because of this, but the physiologically active part is not affected and the fall has no clinical significance.

**Increase in serum calcium** is uncommon. The highest values are found in *hyperparathyroidism*, in which the serum calcium may reach and even sometimes slightly exceed, 20 mg. per 100 ml. In about 25 per cent. of cases, however, it is below 12 mg. per 100 ml. (Herbert, 1951). This rare condition is due almost exclusively to the presence of a parathyroid tumour. The calcium comes from the bones which are extensively decalcified. Injections of parathyroid hormone also raise the serum calcium. There is also increased removal of calcium from the bones in hyperthyroidism, but although, as in hyperparathyroidism, there is increased excretion of calcium in the urine, the serum calcium remains within normal limits in almost all cases. *Hypervitaminosis D*, resulting from excessive dosage with vitamin D preparations, may also raise the serum calcium. The author has seen cases with serum calcium up to 17 mg. per 100 ml. A proportion of cases of *multiple myeloma* are found to have a moderately raised serum calcium which is then usually between normal and 17 mg. per 100 ml. Although the plasma proteins are sometimes increased in this condition, the correlation between the increase in the serum calcium

and increase in plasma proteins is much less close than that with the increase in calcium excretion in the urine. The rise in serum calcium appears to be due to increased removal from the bones.

**Normal serum calcium** is found in all the blood diseases, that is in the various anæmias, purpura, and hæmophilia, except that a small increase may occur in polycythæmia. Normal values are the rule in all bone diseases other than those given above.

## CALCIUM EXCRETION

### Urine Calcium

#### Sulkowich's Test for Urine Calcium

Sulkowich (Barney and Sulkowich, 1937, Albright, 1939) has suggested a test which gives a rough idea of the amount of calcium present in the urine. Carry out the test on a twenty-four-hour specimen of urine if possible.

**Sulkowich's Reagent.** Dissolve 2·5 grams of oxalic acid and 2·5 grams of ammonium oxalate in about 100 ml. of water to which 5 ml. of glacial acetic acid has been added and make up to 150 ml. with water.

**Technique.** Mix thoroughly, equal volumes of urine and Sulkowich's reagent in a test tube and stand for two to three minutes. Calcium present is precipitated as calcium oxalate. The following results may be obtained.

1. No precipitate. No calcium present. Serum calcium probably below 8·5 mg. per 100 ml.
2. Fine white cloud. Moderate urine calcium. Serum calcium probably normal.
3. Heavy milky precipitate shows increased urine calcium, with serum calcium probably above 12·5 mg. per 100 ml.

#### Determination of Urine Calcium

1. **McCrudden's Method** (McCrudden, 1911-12)

**Reagents.** 1. Ammonia, S.G. 0·88.
2. Concentrated hydrochloric acid.
3. Oxalic acid, 2·5 per cent. solution.
4. Sodium acetate, 20 per cent. solution. Use the crystalline salt.
5. Sulphuric acid, approximately 2 N solution.
6. N/10 potassium permanganate.

**Technique.** If necessary, make the urine slightly acid to litmus and filter. Then measure 200 ml. into a suitable flask, make neutral to litmus with ammonia, and add 10 drops of concentrated hydrochloric acid. Add 10 ml. of 2·5 per cent. oxalic acid followed by 8 ml. of 20 per cent. sodium acetate added slowly with shaking. Allow to stand, either overnight, or for about half an hour with frequent vigorous shaking. Filter, wash well with distilled water, dissolve the precipitate in warm 2 N sulphuric acid and titrate with N/10 permanganate.

**Calculation.** 1 ml. of N/10 permanganate is equivalent to 2 mg. of calcium or 2·8 mg. of calcium oxide. Hence

$$\text{Mg. calcium excreted daily} = \text{ml. of N/10 permanganate required} \times 2 \times \frac{\text{Volume of twenty-four hour urine}}{200}$$

$$= \frac{\text{ml N/10 permanganate required} \times \text{Volume of twenty-four hour urine}}{100}$$

Mg. calcium per 100 ml. of urine = ml. N/10 permanganate required.

### 2. Method of Shohl and Pedley (1922)

This is rather more troublesome, but is more accurate.

**Reagents.** 1. Ammonium persulphate.
2. Oxalic acid, 2·5 per cent. solution.
3. Methyl red indicator.
4. N/20 potassium permanganate.
5. Concentrated nitric acid.
6. Concentrated sulphuric acid.
7. Ammonia, S.G. 0·88.
8. Dilute sulphuric acid.

**Technique.** Add 5 ml. concentrated nitric acid or sulphuric acid and 3 to 4 grams of ammonium persulphate to 100 ml. of unfiltered urine in a 250 ml. flask. Insert a filter funnel into the neck of the flask to prevent loss by spattering. Boil gently or keep near to boiling for an hour until reduction of the persulphate is complete, as shown by absence of frothing when the flask is shaken. The solution is pale green in colour. Add 10 ml. of 2·5 per cent. oxalic acid. Cool to room temperature and neutralize with ammonia using 1 drop of methyl red indicator. Stand overnight. Filter through a hardened filter paper (Whatman No. 50 is satisfactory). Wash the precipitate and flask three times with distilled water. Make a hole in the filter paper and wash the precipitate into the original flask first with distilled water and then with dilute sulphuric acid. Warm to about 70° C. and titrate with the N/20 permanganate to the first pink colour which persists for fifteen to thirty seconds.

**Calculation.** One ml. of N/20 permanganate is equivalent to 1 mg. of calcium. Hence

Mg. calcium per 100 ml. urine = ml. permanganate used in the titration.

## Calcium in the Fæces

### Determination of Fæcal Calcium

Dry the fæces, weigh, and ash a portion of about 1 gram in a crucible in an electric furnace. Dissolve the ash in dilute acid and estimate as in McCrudden's method, that is, make neutral to litmus with ammonia, add 10 drops of concentrated hydrochloric acid, 10 ml. of 2·5 per cent. oxalic acid, 8 ml. of sodium citrate, and proceed as described above.

**Calculation.** Mg. calcium in the specimen = ml. permanganate required $\times 2 \times \frac{\text{total weight of dried fæces}}{\text{weight of fæces used in the estimation}}$.

CALCIUM EXCRETION. INTERPRETATION

Most of the calcium excreted from the body is in the fæces, so that normally nearly three times as much is lost in the fæces daily as in the urine. Since in adults the total calcium excreted is likely to average 0·7 to 0·8 gram, the daily excretion of calcium in the urine is normally in the region of 200 mg., that in the fæces about 600 mg. (as Ca). Naturally this is greatly influenced by the amount of calcium in the diet which may vary widely, being largely dependent on the quantity of calcium-rich foods such as milk (680 mg. per pint) and cheese (230 mg. per ounce) which are being consumed.

Estimation of urine calcium is not very frequently made. It is low in diseases in which calcium absorption from the intestine is defective, and so is low in *rickets* whereas the fæcal calcium, so long as calcium intake is normal, is rather high.

There is an increased excretion of calcium in the urine in *hyperparathyroidism* in which well over 300 mg. of calcium may be lost daily in the urine. There is also increased excretion in hyperthyroidism. Greatly increased amounts may also be excreted in *multiple myeloma*. The author has seen several cases with a daily excretion between 300 and 500 mg., and one of 750 mg.

**Calcium balance tests** are sometimes carried out. The patient is placed on a diet of known calcium content. After allowing at least three days for the patient to become adjusted, give a marker before breakfast on the day the collection of twenty-four-hour urine specimens is begun. Continue these collections for several days, say four days, at the end of which period a second marker is given again before breakfast, the point at which urine collection ends. Fæces are collected from marker to marker, including in the test the specimen which contains the first marker but not that which contains the second. As markers, charcoal, carmine, and gentian violet have been used. The dyes are more satisfactory, but it must be acknowledged that marking the fæces accurately is difficult. Furthermore, it is not easy to carry out reliable calcium balance tests under ordinary hospital conditions. Determine the calcium content of the urine and fæces, calculate the average daily excretion, and compare this with the known daily intake.

A normal diet may contain about 800 mg. of calcium daily, and such diets have been used for the test. On this a normal person should be in a state of calcium balance with intake and output about the same. If more calcium is excreted than is taken the patient is said to have a negative calcium balance, and vice versa. Diets low in calcium have also been used in the test. A patient on a diet containing only 100 mg. calcium will normally excrete about 250 mg. calcium daily since a state of balance is not reached until at least 500 mg. calcium are being taken. With such a calcium intake a negative calcium balance exists when more than 250 mg. calcium are being excreted daily, a positive balance when the excretion is less than 250 mg. This form of test is preferred since it is less subject to errors in carrying it out.

There is a negative calcium balance in hyper-parathyroidism. The

test has been said to be of diagnostic value in this condition in that there may be a negative balance when serum calcium and inorganic phosphorus are within normal limits.

## INORGANIC PHOSPHORUS

**Determination of Inorganic Phosphorus in Serum and Plasma** (Fiske and Subbarow (1925) )

The filtrate obtained after removing proteins by means of trichloracetic acid is treated with an acid-molybdate reagent which reacts with inorganic phosphate to form phosphomolybdic acid. The hexavalent molybdenum of the phosphomolybdic acid is reduced by means of 1.2.4. aminonaphtholsulphonic acid to give a blue compound which is estimated colorimetrically. The reducing agent has only a negligible effect on the hexavalent molybdenum in the uncombined molybdic acid.

**Reagents.** 1. Trichloracetic acid, 10 per cent. (w/v). Dissolve 10 grams in water and make up to 100 ml. The quality of the trichloracetic acid is important.

2. Sulphuric acid, 10 N. Add 450 ml. of concentrated sulphuric acid slowly whilst cooling to 1,300 ml. of distilled water. Dilute some of this 1 in 10 and titrate with N sodium hydroxide, and make any necessary adjustment in the original.

3. Molybdate I. 2·5 per cent. ammonium molybdate in 5 N sulphuric acid. Dissolve 25 grams of the salt in about 200 ml. of water, transfer to a litre flask containing 500 ml. of 10 N sulphuric acid. Dilute to 1 litre with water.

4. Molybdate II. 2·5 per cent. ammonium molybdate in 3 N sulphuric acid. Make as molybdate I except that 300 ml. of the acid is used instead of 500 ml.

5. Sodium bisulphite, 15 per cent. solution. This must be free from turbidity when used. Freshly prepared solutions may not filter clear and may need to stand for two or three days.

6. Sodium sulphite, 20 per cent. solution. Dissolve 200 grams of $Na_2SO_3,7H_2O$ in 380 ml. of water. Filter from any suspended matter, and keep well stoppered.

7. 0·25 per cent. 1.2.4. aminonaphtholsulphonic acid. Add 0·5 gram of the dry powder to 195 ml. of 15 per cent. sodium bisulphite and 5 ml. of the 20 per cent. sodium sulphite. Stopper and shake until it is dissolved. If the bisulphite solution is old, more than 5 ml. of sulphite may be needed, in which case add more sulphite 1 ml. at a time, shaking well after each addition until complete solution is obtained. Store in the cold in a brown bottle. The solution keeps up to four weeks, and is more stable the higher the acidity so that no more sulphite should be added than is needed to dissolve the aminonaphtholsulphonic acid.

8. Standard phosphate solution. Dissolve 0·351 gram of pure potassium dihydrogen phosphate in water in a litre flask, add 10 ml. of 10 N sulphuric acid and make to the mark with water. Five ml. contain 0·4 mg. phosphorus. The solution keeps indefinitely.

9. Dilute standard for use. Dilute the stock standard 1 to 10. One ml. contains 0·008 mg. phosphorus.

**Technique.** Pipette 8 ml. of 10 per cent. trichloracetic acid into a suitable test tube and run in 2 ml. of serum or plasma whilst shaking constantly. Use 9 ml. of acid and 1 ml. of serum, or half quantities throughout, if it is desired to economize serum. Mix well, stand for a few minutes and filter through an ashless filter paper. Pipette 5 ml. of the filtrate into a 10 ml. stoppered graduated cylinder. Add 1 ml. of molybdate II reagent and mix. Then add 0·4 ml. of the aminonaphtholsulphonic acid and make to the mark with distilled water. At the same time put up a standard. Pipette 5 ml. of the standard phosphate solution, equivalent to 0·04 mg. P., into a similar cylinder, and add 1 ml. of molybdate I reagent and 0·4 ml. of aminonaphtholsulphonic acid. Mix gently after each addition and make to 10 ml. After standing five minutes compare in the colorimeter or read in the photoelectric colorimeter, using a red filter or transmission at 680 millimicrons.

**Calculation.** Since 5 ml. of standard contain 0·04 mg. P. and 5 ml. of filtrate is equivalent to 1 ml. of serum, for the photoelectric colorimeter,

Mg. inorganic phosphorus per 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}04 \times \frac{100}{1}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 4.$$

A standard curve can be prepared as follows, using as standard a solution prepared by diluting the stock standard 1 in 4 (1 ml. = 0·02 mg. P.).

| Mg. inorganic phosphorus per 100 ml. serum . . . | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| Ml. standard solution (1 ml. = 0·02 mg. P) . . . | 0 | 0·5 | 1·0 | 1·5 | 2·0 | 2·5 | 3·0 | 3·5 | 4·0 |

Add water to about 7 ml., then 1 ml. of molybdate I and 0·4 ml. of aminonaphtholsulphonic acid, make to 10 ml. and read as above.

The standard corresponding to 4 mg. P per 100 ml. serum should be put up with each batch. For values higher than 8 mg. per 100 ml. use 1 ml. of serum and 9 ml. of acid. Even less, 0·5 ml. with 9·5 ml. acid can be used for very high levels.

**Notes.** 1. The lower acid content of the molybdate used for the blood filtrate is to compensate for the trichloracetic acid in it.

2. *Choice of Blood Specimen.* The determination should be made on either serum or plasma separated soon after withdrawing the blood. The inorganic phosphorus increases when blood is allowed to stand, since there is then breakdown of ester phosphates present in the red cells.

3. King has used a slightly different aminonaphtholsulphonic acid reagent which contains 0·2 mg. together with 12 grams of sodium

metabisulphite and 2·4 grams of sodium sulphite ($Na_2SO_3$, $7H_2O$) in 100 ml. of solution, filtered if neeessary, and prepared freshly every fortnight. Messrs. Gallenkamp supply tablets of which one tablet makes 10 ml. of this solution (King and Allott, 1952).

4. *Use of Metol as Reducing Agent.* Gomorri (1942) suggested the use of metol instead of aminonaphtholsulphonie acid. He uses a solution prepared by dissolving 1 gram of metol (*p*-methylaminophenol sulphate) in 100 ml. of a 3 per eent. solution of sodium bisulphite. This reagent is easier to prepare, keeps better at ordinary temperatures, and is less affeeted by variations in the amount of acid present, than the aminonaphtholsulphonic acid. Only one molybdate reagent is required. This is prepared by dissolving 7·5 grams of ammonium molybdate in about 200 ml. of water, adding 100 ml. of 10 N sulphurie aeid and making to 400 ml. with water. Otherwise the determination can be carried out as described above with these two reagents substituted and using 1 ml. of the metol solution. The author has found this quite a satisfactory method.

**Determination of Inorganic Phosphorus on the Supernatant Fluid Obtained after Removing Calcium** (Jones, 1951)

Jones has determined inorganic phosphorus, using the above Gomorri method, on the supernatant fluid from eentrifuging the precipitate of calcium oxalate in the determination of calcium given earlier (p. 293). This may be useful when insuffieient serum is available for both determinations, though it is not quite as aeeurate as the straight determination. After eentrifuging down the precipitate of calcium oxalate, pipette off 2·5 ml. of the supernatant fluid into a test tube containing 7·5 ml. of 10 per eent. trichloraeetie acid. Mix well and filter. To 5 ml. of the filtrate (= 0·5 ml. of serum) add 2·5 ml. of the molybdate reagent, and 1·25 ml. of N/10 potassium permanganate. Shake well and heat for at least five minutes in boiling water, cool, add 1 ml. of the metol solution and make up to 10 ml. with water. If any turbidity develops, eentrifuge and use the supernatant fluid. Read in the colorimeter as before. The dilution is 1 in 20 instead of 1 in 10 in the method described above.

**Method of Kuttner and Lichtenstein** (Kuttner and Lichtenstein, 1930 ; Shinowara, Jones and Reinhart, 1942)

Kuttner and Lichtenstein used stannous chloride as reducing agent. The method is somewhat simpler than the above, in that the stoek stannous chloride solution keeps almost indefinitely in contrast to the aminonaphtholsulphonic aeid, and gives a deeper colour. However, the colour produced appears to be rather variable with time. The method has been widely used although it is not quite so reliable as that of Fiske and Subbarrow. With care, however, it should give results accurate enough for clinical purposes.

**Reagents.** 1. Trichloraeetie acid, 10 per eent. (w/v).

2. Sulphuric acid, 10 N. Pour 282 ml. of eoncentrated acid into about 600 ml. of distilled water, cool, and make up to 1 litre.

3. Sodium molybdate, 7·5 per cent. solution. Remove by decanting or filtering, any deposit which settles out.

4. Sulphuric acid-sodium molybdate mixture. Mix 1 volume of 10 N sulphuric acid with 2 volumes of water, cool, and pour into 1 volume of 7·5 per cent. sodium molybdate. Prepare freshly at frequent intervals.

5. Stock stannous chloride solution. Forty per cent. in concentrated hydrochloric acid. Dissolve 10 grams of stannous chloride in 25 ml. of acid. Although this keeps well it is recommended that it be prepared freshly every month or two.

6. Dilute stannous chloride solution. Dilute 1 ml. of the stock solution to 200 ml. with distilled water. Prepare freshly before use.

7. Standard phosphate solution. One ml. contains 1 mg. phosphorus. Dissolve 4·394 grams of potassium dihydrogen phosphate in 1 litre of distilled water and add a few drops of chloroform.

8. Standard phosphate for use. Dilute the stock solution 1 in 100 before use, so that 1 ml. contains 0·01 mg. of phosphorus.

**Technique.** Add 1 ml. of plasma or serum to 4 ml. of 10 per cent. trichloracetic acid in a test tube. Mix well and filter. Take 2 ml. of the filtrate and add to it 5 ml. of distilled water, 2 ml. of the sulphuric acid-molybdate reagent, and 1 ml. of dilute stannous chloride solution. Mix. Treat 2 ml. of the phosphate standard similarly. Compare in the colorimeter after a few minutes, using a red filter or transmission at 680 millimicrons with the photoelectric colorimeter.

**Calculation.** Since 2 ml. of filtrate corresponds to 0·4 ml. of serum, and 2 ml. of standard contain 0·02 mg. phosphorus, for the photoelectric colorimeter,

Mg. inorganic phosphorus per 100 ml. serum or plasma

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}02 \times \frac{100}{0{\cdot}4}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 5.$$

A standard curve can be prepared using the dilute standard :

| Mg. inorganic phosphorus per 100 ml. | 0 | 1·0 | 2·0 | 3·0 | 4·0 | 5·0 | 6·0 | 8·0 | 10·0 |
|---|---|---|---|---|---|---|---|---|---|
| Ml. standard solution (1 ml. = 0·01 mg. P) | 0 | 0·4 | 0·8 | 1·2 | 1·6 | 2·0 | 2·4 | 3·2 | 4·0 |
| Ml. water | 7·0 | 6·6 | 6·2 | 5·8 | 5·4 | 5·0 | 4·6 | 3·8 | 3·0 |

Add 2 ml. molybdate reagent and 1 ml. stannous chloride, and read as for the test.

For higher values it is better to use a smaller volume of the trichloracetic acid filtrate. Study whether Beer's law is followed satisfactorily.

INTERPRETATION

The normal plasma or serum inorganic phosphorus is between 2·5 and 4·5 mg. per 100 ml. in most adults. It is higher in children, whose normal range is from 4 to 6 mg. per 100 ml.

An *increase* in serum inorganic phosphorus is found in *chronic nephritis*, rising progressively with increasing renal failure, and reaching a level in the vicinity of 20 mg. per cent. in the final stages when uræmic coma is present. There is a moderate increase in *hypoparathyroidism* in which the low serum calcium levels are associated with values for serum inorganic phosphorus between 4 and 7 mg. per 100 ml.

As mentioned above, serum inorganic phosphorus is usually *reduced* in *rickets*, in untreated cases of which it may be down as low as 2 mg. per 100 ml., rising on treatment. It also tends to be rather low in *hyperparathyroidism* when it may be in the region of 2 mg. per cent., but this fall is not so marked from the normal adult level as is the fall in rickets from the normal level for children. In *osteomalacia*, adult rickets, figures of below 2 mg. per 100 ml. are observed.

There is a normal physiological fall to below 3 mg. per 100 ml. when there is increased carbohydrate utilization. Injection of insulin has the effect of reducing the serum inorganic phosphorus. The very low levels which may occur during the treatment of *diabetic coma*, when values below 1 mg. per cent. may result, arise from this cause.

Serum inorganic phosphorus is within normal limits in all bone conditions other than those mentioned above.

## BLOOD PHOSPHORUS

It will be convenient to introduce here a little about the various phosphorus compounds present in blood. We have already dealt with inorganic phosphorus in this chapter and with lipid phosphorus in Chapter XII (p. 190). In addition there are present in blood, organic phosphate esters and some residual phosphorus-containing compounds. Accordingly the blood phosphorus may be classified :

1. Inorganic phosphorus,—the phosphates of the alkali and alkaline earth metals.
2. Organic or ester phosphorus, including glycerophosphates, nucleotide phosphates, hexosephosphate, and others.
3. Lipid phosphorus—lecithin, cephalin, sphingomyelin.
4. A small amount of residual phosphorus.

The whole is referred to as total phosphorus, the first two together as acid soluble phosphorus. The values for the various components may be summarized as follows (Simonsen *et al.*, (1946) ; Stearns and Warweg (1933) ) :

| | Whole blood | Plasma or serum |
|---|---|---|
| Total phosphorus (as P) . | 30–50 | 10 –15 mg. per 100 ml. |
| Inorganic phosphorus . . | 2– 5 | 2·5– 4·5 ,, ,, |
| Ester phosphorus . . | 15–30 | 0 – 1 ,, ,, |
| Acid-soluble phosphorus . | 20–30 | 3 – 6 ,, ,, |
| Lipid phosphorus . . | 10–16 | 6 –11 ,, ,, |

It is seen that ester phosphorus is much more plentiful in the cells than in the plasma in which it is present in only small amounts.

## Total Acid Soluble Phosphorus

### Determination

Total acid-soluble phosphorus is determined by digesting some of the trichloracetic acid filtrate with sulphuric acid and hydrogen peroxide and estimating the inorganic phosphate thus formed by the method of Fiske and Subbarow.

**Technique.** Using *whole blood*, take 2 ml. of the trichloracetic acid filtrate as prepared for estimating inorganic phosphorus and add 1·5 ml. of 10 N sulphuric acid. Digest over a small flame. When the mixture is brown, remove the flame, allow the tube to cool and add 1 drop of 30 per cent. hydrogen peroxide. Heat again, repeating this process until the mixture is colourless. Then cool, add a few ml. of water and bring to the boil. After cooling, transfer to a 25 ml. volumetric flask bringing to about 15 ml. with water. Add 2·5 ml. of 2·5 per cent. ammonium molybdate and 1 ml. of aminonaphthol-sulphonic acid. Make up to the mark, mix, and after standing five minutes, read in the colorimeter. Carry through a blank and standard, digesting and developing the colour as for the test. For the blank use water instead of the blood specimen, for the standard a volume containing 0·1 mg. of phosphorus (1·25 ml. of the stronger standard on p. 302, or 1 ml. of a solution containing 0·439 gram of pure potassium dihydrogen phosphate in a litre of solution containing 10 ml. 10 N sulphuric acid.)

**Calculation.** Two ml. of filtrate correspond to 0·4 ml. of the blood specimen and the standard contains 0·1 mg. phosphorus. So, using the photoelectric colorimeter

Mg. acid soluble phosphorus per 100 ml.

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}1 \times \frac{100}{0{\cdot}4}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 25.$$

Use the blank to set the colorimeter or read against the blank in a double-cell instrument.

The above is satisfactory for whole blood. For *serum* take 4 ml. of trichloracetic acid filtrate and make up to 10 ml. instead of 25 ml. when developing the colour. Put up a standard containing 0·04 mg. of phosphorus and make up to a final volume of 10 ml. The calculation then becomes

Mg. acid soluble phosphorus per 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 5.$$

## Phosphorus Excretion

### Determination of Inorganic Phosphorus in Urine

The methods used for serum and plasma can be applied to urine.

### Method of Fiske and Subbarrow (1925)

**Reagents.** These are the same as for serum (p. 302).

**Technique.** Dilute the urine 1 in 10 unless it is a very dilute one, when a dilution of 1 in 5 should be used. Pipette 1 ml. of diluted urine into a 10 ml. stoppered measuring cylinder. The best amount of urine to use is that which contains between 0·02 and 0·08 mg. inorganic phosphorus. Add water to about 7 ml. followed by 1 ml. of molybdate reagent I, and then 0·4 ml. of 0·25 per cent. amino-naphtholsulphonic acid. Mix gently after each addition. Finally, make up to the mark with water. Prepare a standard at the same time, using 5 ml. of standard containing 0·04 mg. of phosphorus, and treat in the same way as the unknown. Compare in the colorimeter as before.

**Calculation.** Using 1 ml. of urine diluted 1 in 10, for the photo-electric colorimeter:

Mg. inorganic phosphorus per 100 ml. of urine

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}04 \times \frac{100}{0{\cdot}1}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 40.$$

For a dilution of 1 in 5 the factor is 20.

The standard curve prepared for the serum determination can be used, bearing in mind that the urine is diluted ten times more than the serum, so that the result as read from the curve must be multiplied by 10 for urine diluted 1 in 10, and by 5 for urine diluted 1 in 5.

**Note.** If proteins are present in the urine so that addition of the molybdate causes turbidity, they must be removed. This can be done by adding 10 per cent. trichloracetic acid. Take 4 volumes of this to 1 volume of urine, mix well and filter. Use an amount of filtrate between 0·5 and 1 ml. equivalent to 0·1 to 0·2 ml. of urine and proceed as above.

#### INTERPRETATION

The amount of phosphorus excreted in the urine naturally depends mainly on the amount of phosphorus taken in the diet. The daily excretion on an average diet is about 1 gram (as phosphorus). This is nearly all inorganic phosphate. The inorganic phosphates belong to three types according to whether one, two or three of the hydrogen atoms of phosphoric acid are replaced by the metal. Thus we have for sodium and calcium, $Na_3PO_4$, $Na_2HPO_4$, $NaH_2PO_4$; $Ca_3(PO_4)_2$, $CaHPO_4$, $Ca(H_2PO_4)_2$. The dihydrogen salts have an acid reaction, the monohydrogen an alkaline. The dihydrogen salts of calcium and magnesium are more soluble than the monohydrogen so that these latter precipitate more easily when the urine is alkaline than do the dihydrogen when the urine is acid. Such a precipitation is due to altered solubility and need not indicate any increased excretion of phosphates.

Ordinarily phosphorus in the urine is almost entirely inorganic phosphorus. Strictly speaking in *determining the total phosphorus*, organic matter should be destroyed. This can be done by heating the urine with sulphuric acid, 1 ml. of urine with 1 ml. of concentrated sulphuric acid, completing the oxidation with hydrogen peroxide (100 volumes) added drop by drop. When clear, add 2 to 3 ml. of distilled water, boil for two to three minutes, cool, make up to 10 ml. with water, take 1 ml. and proceed as above for inorganic phosphorus.

Estimation of urinary phosphorus is rarely done except as part of metabolism experiments and in research. There is increased excretion of phosphorus in the urine in hyperparathyroidism ; giving parathyroid hormone has a similar effect. Diminished production of parathyroid hormone has the opposite effect so that in hypoparathyroidism there is reduced excretion of inorganic phosphorus. It is also reduced in rickets due to the impaired absorption of phosphorus in that condition. Treatment with either vitamin D or ultraviolet light leads to an increase in the output of phosphorus in the urine.

### Fæcal Phosphorus

The phosphorus in fæces is in both organic and inorganic form. Since an estimation of this would only be carried out in metabolism experiments it is total phosphorus which must be determined. For this purpose it is best to dry the fæces, determine their weight, powder well, weigh out about 1 gram and ash in an electric furnace at a temperature of about 400° C., rather below red heat. Dissolve the powder in 25 ml. of 20 per cent. trichloracetic acid, filter into a 200 ml. flask, washing in with further trichloracetic acid up to 25 ml. and then make up to the mark with water. Take 1 ml. of this solution and estimate the phosphorus content as described under serum inorganic phosphorus.

On an adequate intake of phosphorus, about 1 to 2 grams daily, intake and output balance and about two-thirds of the phosphorus excreted is found in the urine, about one-third in the fæces.

## PHOSPHATASES

Phosphatases are enzymes which catalyze the splitting off of phosphoric acid from certain monophosphoric esters, a reaction of considerable importance in several body processes.

Two types of phosphatase are commonly estimated in serum, an alkaline phosphatase showing maximum activity at *p*H 10 and an acid phosphatase with maximum activity about *p*H 5. The method most commonly used is that of King and Armstrong, in which disodium phenyl phosphate is hydrolyzed with liberation of phenol and formation of sodium phosphate. The amount of phenol so formed is estimated colorimetrically.

## Serum Alkaline Phosphatase

**Determination. Method of King and Armstrong.** (King and Armstrong, 1934 ; King *et al.*, 1937, 1942)

**Reagents.** 1. M/100 disodium phenyl phosphate. Dissolve 1·09 grams in water and make up to 500 ml. Bring quickly to the boil. Cool, add a little chloroform and keep in the refrigerator.

2. M/10 sodium carbonate—sodium bicarbonate buffer. Dissolve 3·18 grams of anhydrous sodium carbonate and 1·68 grams of sodium bicarbonate in water and make up to 500 ml.

3. Buffered substrate for use. Prepare by mixing equal volumes of solutions 1 and 2. This has a *p*H of 10.

4. Phenol reagent of Folin and Ciocalteu (1927). Dissolve 100 grams of sodium tungstate ($Na_2WO_4$, $2H_2O$) and 25 grams of sodium molybdate ($Na_2MoO_4$, $2H_2O$) in about 700 ml. of water in a 2 litre round bottomed flask. Add 50 ml. of syrupy 85 per cent. phosphoric acid and 100 ml. of concentrated hydrochloric acid. Reflux for ten hours. Use all-glass apparatus if possible, otherwise wrap the stopper in tin foil. Add 150 grams of lithium sulphate, 50 ml. of water and a few drops of bromine. Boil without a condenser for about a quarter of an hour to remove the excess of bromine. Cool, make up to a litre, and filter. The solution should have no greenish tint. This is an improved phenol reagent which reduces the possibility of turbidity due to insoluble urates.

Dilute this solution 1 in 3 for use in this determination, that is, to 1 volume of phenol reagent add 2 volumes of water.

5. Twenty per cent. sodium carbonate solution. Dissolve 20 grams of anhydrous sodium carbonate in water and make up to 100 ml. This solution is almost saturated at room temperature and so should be kept in a warm place to prevent the salt from crystallizing out.

6. Standard phenol solution. Stock solution. One hundred mg. of phenol per 100 ml. of solution. Dissolve 1 gram of pure crystalline phenol in N/10 hydrochloric acid and make up to 1 litre with the acid. This can be standardized as follows : place 25 ml. into a 250 ml. flask. Add 50 ml. of N/10 sodium hydroxide and heat to 65° C. To the hot solution add 25 ml. of N/10 iodine. Stopper and allow to stand for thirty to forty minutes. Add 5 ml. of concentrated hydrochloric acid and titrate the excess iodine with N/10 sodium thiosulphate. Each ml. of N/10 iodine is equivalent to 1·57 mg. of phenol.

7. Diluted phenol standard for use. Dilute the stock standard 1 in 10 to obtain a standard solution containing 10 mg. phenol per 100 ml. of solution.

8. Standard phenol solution and reagent, containing 0·5 mg. phenol per 100 ml. Take 2·5 ml. of the dilute standard, add 15 ml. of the diluted phenol reagent and make up to 50 ml. with water. This is best prepared daily.

**Technique.** Pipette 6 ml. of the buffer substrate into a test tube and place in the water bath at 37° C. for a few minutes. Add 0·3 ml. of serum preferably without removing from the bath. Mix and cork and allow to remain in the bath exactly fifteen minutes. Then

remove and immediately add 2·7 ml. of the diluted phenol reagent. At the same time set up a tube for the blank containing 6 ml. of substrate and 0·3 ml. of serum to which is added immediately 2·7 ml. of diluted phenol reagent. Mix well in both cases and filter or centrifuge.

Take 4 ml. of filtrate from each and add 1 ml. of 20 per cent. sodium carbonate. Put up a standard prepared by adding 1 ml. of sodium carbonate solution to 4 ml. of the standard phenol and reagent. Place the three tubes in the 37° C. water bath for fifteen minutes and read in the colorimeter. The method is most conveniently used with the photoelectric colorimeter. A red filter is used, with transmission at 680 millimicrons.

**Calculation.** The result is expressed in mg. of phenol liberated by 100 ml. of serum in fifteen minutes at 37° C., each unit corresponding to the liberation of a mg. of phenol per 100 ml. of serum.

It is seen that for the photoelectric colorimeter

Mg. phenol per 100 ml. unknown

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \text{amount of phenol in standard} \times \frac{100}{\text{amount of serum used}}.$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}02 \times \frac{9}{4} \times \frac{100}{0{\cdot}3}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 15.$$

Units phosphatase per 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 15 \quad - \quad \frac{\text{Reading of blank}}{\text{Reading of standard}} \times 15.$$

For the photoelectric colorimeter a standard curve can be prepared as follows using the diluted phenol standard (7) above :

| Mg. phenol per 100 ml. serum | 0 | 6 | 12 | 18 | 24 | 30 | 36 |
|---|---|---|---|---|---|---|---|
| Ml. dilute phenol standard . | 0 | 0·2 | 0·4 | 0·6 | 0·8 | 1·0 | 1·2 |
| Ml. water . . . | 7·0 | 6·8 | 6·6 | 6·4 | 6·2 | 6·0 | 5·8 |

Add 3·0 ml. of dilute Folin and Ciocalten reagent and 2·5 ml. of 20 per cent. carbonate and proceed as described above.

Read unknown and blank and subtract the mg. phenol per 100 ml. of the latter from that of the former to obtain the units of phosphatase per 100 ml. serum.

**Notes.** 1. In earlier forms of the method, King and Armstrong used a substrate buffered with sodium barbitone. M/200 disodium phenyl phosphate in M/20 sodium barbitone made by dissolving 1·09 grams of disodium phenyl phosphate and 10·3 grams of sodium barbitone in water and making up to a litre is used. A few drops of chloroform were added as preservative, the solution being kept in the refrigerator. The *p*H at which the test was carried out was about

9·0. Incubation was for thirty minutes since phosphatase activity at such a *p*H is only about half that at the *p*H of the carbonate-bicarbonate buffer. By using different times of incubation, however, the normal range is the same in each case. Furthermore, the phosphatase activity is changing in a marked way at a *p*H about 9·0 but has a fairly flat peak in the region of *p*H 10. Small variations in *p*H thus cause much smaller differences in phosphatase activity with the carbonate-bicarbonate buffer.

2. With high serum phosphatase values it is advisable to use less serum, since the reaction ceases to be proportional to the amount of phosphatase present. So for values of more than 40 units per 100 ml. dilute the serum 1 in 5 and repeat. Occasionally it may be necessary to dilute even more. With such a modification, values of 100 to 200 units are not uncommon, and even higher figures may be obtained, though only rarely.

**Determination of Alkaline Phosphatase by Measuring Liberated Inorganic Phosphate** (Method of King, Abul-Fadl, and Walker, 1951)

In some bone diseases, estimation of inorganic phosphate is required as well as alkaline phosphatase, so that it is convenient, if possible, to determine them together. King has adapted his method for alkaline phosphatase so that this can be done.

**Reagents.** 1. Buffer substrate. As for the previous method.

2. Trichloracetic acid, 20 per cent. (w/v).

3. Acid molybdate solution. Dissolve 5 grams of ammonium molybdate in 5N sulphuric acid. (Add 14 ml. of concentrated acid slowly to 86 ml. water to prepare the acid.)

4. 1 : 2 : 4 aminonaphtholsulphonic acid reducing reagent. As for estimation of inorganic phosphate.

5. Stock phosphate standard (1 mg. P. per ml.). Dissolve 2·194 grams of pure potassium dihydrogen phosphate ($KH_2PO_4$) in water, and make to 500 ml. Add a few drops of chloroform.

6. Standard for use. 0·004 mg. P per ml. Dilute 2 ml. of stock standard to 500 ml. (5 ml. = 0·02 mg.).

**Technique.** Measure 6 ml. of buffer substrate into a suitable test tube and place in a bath at 37° C. for a few minutes. Add 0·3 ml. of serum, stopper the tube, mix, and incubate for fifteen minutes. Remove the tube, and at once add 1·2 ml. of 20 per cent. trichloracetic acid. Shake well and filter.

At the same time set up a control and blank. For the blank, use 0·3 ml. serum and 6 ml. of buffer substrate, for the control, 0·3 ml. serum and 6 ml. of water. To each add 1·2 ml. of 20 per cent. trichloracetic acid, mix, and filter.

Pipette 5 ml. of each of the three filtrates, and of the phosphate standard, and add 0·8 ml. of the acid-molybdate followed by 0·2 ml. of the aminonaphtholsulphonic acid. Mix, stand ten minutes, and read in the photoelectric colorimeter using a red filter.

**Calculation.** The inorganic phosphate is given by the control :

Mg. inorganic phosphorus per 100 ml.

$$= \frac{\text{Reading of control}}{\text{Reading of standard}} \times 0{\cdot}02 \times \frac{100}{0{\cdot}2}$$
$$= \frac{\text{Reading of control}}{\text{Reading of standard}} \times 10.$$

The alkaline phosphatase is given by
Units alkaline phosphatase per 100 ml.

$$= \frac{\text{Reading of unknown} - \text{Reading of control} - \text{Reading of blank}}{\text{Reading of standard}} \times 0{\cdot}02 \times 3 \times \frac{100}{0{\cdot}2}, \text{ i.e. } \times 30.$$

The 0·02 is from the mg. P in the standard ; 0·2 is the volume in ml. of plasma in 5 ml. of filtrate ; and the factor 3 is introduced in order that the normal range may be the same as that given for the method estimating the amount of phenol liberated. One phosphate group is liberated and one phenol group. The ratio of P to $C_6H_5OH$ is 31 to 94, so that 1 mg. of phosphorus is very nearly equal to 3 mg. of phenol. Hence the factor 3.

**Note.** King has also published a variation of this method using stannous chloride as the reducing agent. This can be done on only 0·1 ml. of serum.

*Other Methods.* Hydrolysis of sodium β-glycerophosphate at *p*H 8·6 for one hour at 37° C. is used in the method of Bodansky (Bodansky, 1932, 1933, 1937 ; see also Shinowara *et al.*, 1942). The amount of inorganic phosphorus liberated is measured. This method has been widely used, particularly in America. Each unit corresponds to 1 mg. of inorganic phosphorus liberated per 100 ml. of serum. The normal range is 1·5 to 4 units per 100 ml. for adults, and up to 12 units per 100 ml. for children.

Huggins and Talalay (1945) used hydrolysis of sodium phenolphthalein phosphate, determining the amount of hydrolysis by making alkaline. Bessey *et al.*, (1946) hydrolyzed *p*-nitrophenyl phosphate, measuring the amount of yellow *p*-nitrophenol liberated. These methods are described by Milton and Waters (1949).

INTERPRETATION

Alkaline phosphatase is found in a number of organs, being most plentiful in the small intestine, bones and kidneys. The normal range for the serum alkaline phosphatase by the King-Armstrong method is from 3 to 13 units per 100 ml., with rather higher values for children, particularly during periods of most active growth, when values up to 30 units are found.

Increases are observed in certain bone diseases and in some types of jaundice. The increases in jaundice and liver disease have already been discussed, p. 257.

In bone disease serum alkaline phosphatase activity is increased when bone regeneration is taking place, or being attempted. It is not found when there is bone destruction unless there is simultaneous formation of new bone or osteoid tissue. Thus there is marked

increase in *rickets*, in which there is frustrated attempt at bone formation, the increases roughly paralleling the severity of the condition. Values of more than 100 units may be obtained. There is also an increase in *osteomalacia*, adult rickets, but it is not usually so marked as in children. In *osteitis deformans* (Paget's disease), particularly high figures are seen, arising from the abnormal proliferation of bone which is occurring. The serum level often exceeds 100 units per 100 ml. and on occasion may be over 200 units. In one case the author found a value of 550 units per 100 ml. In *hyperparathyroidism* the bone destruction is more extensive than the bone regeneration, the reverse of the state of affairs in Paget's disease, so that although there is some increase in the serum alkaline phosphatase, this is not usually so marked and is mostly found to be in the range 20 to 70 units, but with occasional findings up to about 100 units.

In *bone tumours* the findings are variable, depending on the amount of bone formation taking place. Marked increases may be found in osteogenic sarcomas but in other cases, normal or nearly normal results are obtained. In the patients with secondary deposits in bones, increases may occur, sometimes appreciable. In *multiple myeloma* in which the lesion is almost purely destructive, serum alkaline phosphatase is usually within normal limits, with never more than very small increases.

In the *steatorrhœas* the impaired absorption of vitamin D and of calcium leads to bone changes, and to small to moderate increase in serum alkaline phosphatase, usually to between 20 and 60 units per 100 ml.

It is thus seen that although increases tend to be greater in some conditions than in others, alkaline phosphatase estimation alone is of limited value in bone disease. For differential diagnosis it is necessary to take the findings in conjunction with those for other constituents of serum, such as calcium, inorganic phosphorus and proteins.

## Serum Acid Phosphatase

**Determination** (Gutman and Gutman, 1938, 1940)

The King-Armstrong method for alkaline phosphatase was adapted for the estimation of acid phosphatase by Gutman and Gutman, who substituted a different buffer so that the reaction could be carried out at *p*H 4·9.

**Reagents.** 1. M/100 disodium phenyl phosphate. Dissolve 1·09 grams in water and make up to 500 ml.

2. Citric acid—sodium citrate buffer. Dissolve 21 grams of crystalline citric acid in water and add 188 ml. of normal sodium hydroxide and make up to 500 ml. with water. Adjust to *p*H 4·9 by adding sodium hydroxide or hydrochloric acid.

Alternatively, dissolve 29·41 grams of sodium citrate, $C_6H_5O_7Na_3$, $2H_2O$, in 0·2 N hydrochloric acid and make up to 500 ml. with the acid.

Keep solutions 1 and 2 in the refrigerator.

3. Buffer substrate. Prepare freshly for use by mixing equal quantities of the two solutions given above.

The other solutions required are the same as for the estimation of serum alkaline phosphatase.

**Technique.** The method is almost the same as that given for serum alkaline phosphatase. Use 6 ml. of the buffer substrate with 0·3 ml. of serum and incubate for one hour at 37° C. Subsequent procedure is identical with that given previously.

**Calculation.** The result in this case is expressed in terms of the mg. of phenol liberated by 100 ml. serum in one hour at 37° C., each unit corresponding to 1 mg. of phenol thus formed. The calculation is thus exactly the same as that given for alkaline phosphatase.

**Notes.** 1. Serum for the estimation should be separated without hæmolysis (see below) and kept frozen in the refrigerator if the estimation cannot be carried out on the day the blood was taken, which is preferable. Prostatic acid phosphatase is heat labile and becomes inactivated if the serum is kept at 37° C. for an hour.

2. The reading of the blank ultimately tends to rise on keeping the solutions, usually becoming noticeable after some two to three weeks. If this occurs fresh solutions must be made.

3. As with the alkaline phosphatase, if high values are obtained the estimation should be repeated, using diluted serum. This is best done with values of more than about 40 units per 100 ml., otherwise high values will be useless for comparative purposes. Dilution should be such as will bring the reading to below this level.

4. In the original method the time of incubation was three hours. It is, however, more convenient to use one hour. The method is then a little less accurate for low values and the upper limit of normal is a little higher.

**Determination of Alcohol Stability.** Herbert (1946) showed that whereas the acid phosphatase normally present in serum is not affected by alcohol, that produced by prostatic tissue is inactivated if the serum is allowed to stand with added alcohol. To determine the amount of alcohol stable acid phosphatase add 0·12 ml. of absolute ethyl alcohol to 0·3 ml. of serum and allow to stand for thirty minutes. Then add 6 ml. of the buffer substrate, incubate for one hour and proceed as before. By determining the acid phosphatase both with and without added alcohol we can measure the amount of acid phosphatase derived from the prostate and also that derived from those sources which produce the acid phosphatase present in normal serum. The use of this will be referred to below.

**The Effect of Formaldehyde.** Red blood cells contain considerable quantities of an acid phosphatase so that serum used for this estimation must not contain hæmolysed red cells. Furthermore, both prostatic and red cell acid phosphatase are inactivated by alcohol. Abul Fadl and King (1948) showed that it is possible to differentiate between the red cell acid phosphatase and that arising from prostate tissue. Red cell acid phosphatase is completely inactivated if 0·15 ml. of 20 per cent. neutral formaldehyde is included in the buffer substrate-serum mixture. Alternatively, include 2·5 ml. of 40 per

cent. formaldehyde in 100 ml. of the acid buffer. Prostatic acid phosphatase is not affected. Acid phosphatases from other tissues which contribute to the small amount of acid phosphatase normally present in the serum are variably inactivated by the formaldehyde. So if a high acid phosphatase is obtained after treatment with formaldehyde the evidence is strongly in favour of its prostatic origin which can be further confirmed by the alcohol treatment.

INTERPRETATION

Acid phosphatases which contribute to the normal acid phosphatase present in plasma are found in a number of tissues, such as bone, kidney, spleen, liver, pancreas, There is a large amount in adult prostate tissue and in the prostatic secretion.

The normal serum acid phosphatase ranges from 1 to 5 units per 100 ml. This is non-prostatic in origin and is found in the serum in both sexes at all ages.

The estimation is carried out in connection with malignant disease of the prostate. It has been found that in some cases of malignant prostate with secondaries (in bone) the serum acid phosphatase may be increased, sometimes very greatly. Some illustrative finds are those of Herbert (1946) and of Sullivan *et al.*, (1942). These are tabulated below, in Table XVII.

TABLE XVII.

*Variations in Acid Phosphatase in Disease of the Prostate*

| | | | | | | |
|---|---|---|---|---|---|---|
| 1. *Herbert* (1946) | 1–5 | 5–10 | 10–20 | 20–50 | 50–100 | Over 100 |
| Carcinoma with bone metastases. 35 cases | 6 (17%) | 8 (23%) | 7 (20%) | 4 (11%) | 6 (17%) | 4 (11%) |
| Carcinoma without bone metastases. 47 cases | 35 (74%) | 8 (17%) | 3 (7%) | 1 (2)% | | |
| Senile hypertrophy. 95 cases | 93 (98%) | 2 (2%) | | | | |
| 2. *Sullivan et al.* (1942) | 0–3 | 3–4·9 | 5–9·9 | 10–100 | 100–1000 | Over 1,000 |
| (Three hours incubation.) | | | | | | |
| Carcinoma with bone metastases | 15% | 12% | 25% | 35% | 9% | 4% |
| Carcinoma without bone metastases | 89% | 11% | | | | |

It will be seen that some very high values for acid phosphatase may be found in such patients. Values of over 1,000 units have been reported. That such an increase is due to prostatic acid phosphatase, as we have seen, can be shown with a high degree of certainty if the activity is almost unaffected by formaldehyde (though badly hæmolysed sera should not be used) and if the enzyme is almost completely inactivated by alcohol.

Determination of the acid phosphatase both with and without addition of alcohol and formaldehyde as described above is of particular value in those cases in which the increase of acid phosphatase is small, in the author's experience in the range 5 to 13 units. If the increase is due to prostatic acid phosphatase the alcohol stable fraction will be appreciably lower than the total acid phosphatase, being usually within normal limits, for example :

Total serum acid phosphatase, 8·2 units per 100 ml.

Alcohol stable acid phosphatase, 4·5 units per 100 ml.

On the other hand, small increases in serum acid phosphatase up to about 12 units per 100 ml. are found in diseases such as Paget's disease of bone, in which there is a very high alkaline phosphatase. In such cases the whole of the serum acid phosphatase is found to be alcohol stable.

It should be noted that the agreement between total and alcohol stable acid phosphatase in non-prostatic cases is not exact and the difference may be as much as ± 1 unit per 100 ml. If the total serum acid phosphatase is within normal limits it is very doubtful if any conclusion should be drawn from a difference between the total and alcohol stable.

Determination of serum acid phosphatase may then be of value in establishing a diagnosis of metastasising prostatic carcinoma. The realization that such secondary deposits are of mature prostate tissue, and the known connection of prostate development with male sex hormone production, led to the treatment of the condition with œstrogens, particularly with the synthetic product stilbœstrol, with the object of neutralizing the effect of the male sex hormones, or by orchidectomy, to diminish the output of such hormones. These treatments have met with an undoubted measure of success. They lead, if effective, to a fall in the serum acid phosphatase, estimation of which is accordingly used in helping to assess the effect of such treatment. It should be noted that the alkaline phosphatase, which is frequently raised in these cases of metastasing prostate carcinoma, though usually only moderately, often rises further on successful treatment being initiated, to fall later if bone repair has been effected. Accordingly estimation of both acid and alkaline phosphatase may yield useful information during the treatment of these cases.

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*CHAPTER XVII*

# IODINE, IRON AND COPPER, SULPHUR, MAGNESIUM

In this chapter we shall consider several inorganic elements which are not so frequently determined as those so far discussed.

## IODINE

Since iodine is present in thyroxine, interest in it is almost entirely concerned with disorders of the thyroid gland. Under normal conditions almost all the blood iodine is in the plasma. Less than a third of this is present as inorganic iodine compounds, the remainder being organic and bound to protein (albumin). Whilst the nature of all this protein-bound iodine is not known, about two-thirds of it appears to consist of thyroxine or compounds which behave similarly. Hence variations in protein-bound iodine are related to changes in activity of the thyroid.

The amount of iodine present in the plasma both in health and disease is so small that the determination is not easy and requires great care. Both total iodine and protein-bound iodine have been determined. A method for protein-bound iodine is given. This is a slight modification of that of Barker (1948). For other methods see McCullough (1934), Trevarrow and Fashena (1935), Taurog and Chaikoff (1946). An extensive bibliography of the literature dealing with iodine determination is given by Rapport and Curtis (1950), who also review in detail the clinical significance of the estimation.

**Determination of Protein-bound Iodine.** Method of Barker, Humphrey, and Soley (1951)

The proteins are precipitated, and after washing, are incinerated at a high temperature in the presence of alkali, and the amount of inorganic iodide formed is determined colorimetrically using the catalytic decolorization of a ceric sulphate solution by arsenious acid.

**Reagents.** 1. Ten per cent. zinc sulphate solution. Dissolve 100 grams of $ZnSO_4$, $7H_2O$ in water and make up to 1 litre.

2. N/2 sodium hydroxide. When 10 ml. of the zinc sulphate solution is diluted to about 60 ml. with water, 10·8 to 11·2 ml. of this solution should be required to give a faint permanent pink colour with phenolphthalein.

3. 4 N sodium carbonate.

4. 2 N hydrochloric acid. Add 200 ml. of concentrated acid to water and dilute to 1 litre.

5. 7 N sulphuric acid. Add 196 ml. of concentrated acid slowly to about 750 ml. of water, cool, and make up to 1 litre.

6. N/50 ceric ammonium sulphate solution. Dissolve 12·65 grams in 500 ml. of water plus 230 ml. of 7 N sulphuric acid, and when clear make up to a litre with water.

7. N/10 sodium arsenite. Dissolve 4·95 grams of arsenious oxide in 25 ml. of N sodium hydroxide with warming. Dilute with about 300 ml. of water and add dilute sulphuric acid until slightly acid to litmus paper (about 4 ml. of the 7 N acid are required). Make up to a litre with water.

8. Iodide standards. Dissolve either 118·1 mg. of sodium iodide or 130·8 mg. of potassium iodide in water and make up to 1 litre. Use only highest purity reagents dried in a desiccator. These solutions contain 100 micrograms of iodine per ml. Prepare a more dilute stock solution by diluting 2 ml. of this to 1 litre. Prepare the standard for use at least weekly, by diluting 10 ml. of the dilute stock standard to 50 ml. This now contains 0·04 microgram iodine per ml. All these solutions keep in the refrigerator.

Make up all the above solutions with double distilled water. If too high a blank is still obtained, it may be necessary to distil the water with such substances as alkali and permanganate. Use reagents of the highest purity with proved low blanks throughout.

**Technique.** Carry out the determination in duplicate. Measure 1 ml. of serum into a Pyrex test tube (125 × 15 mm.), dilute with 7 ml. of distilled water and add 1 ml. of 10 per cent. zinc sulphate. Mix with a narrow glass rod (about 2 mm. in diameter), add 1 ml. of N/2 sodium hydroxide, and mix well. Remove any material adhering to the rod by rotating it against the inner wall of the tube. Centrifuge for ten minutes and pour off the supernatant fluid. Add 10 ml. of distilled water and resuspend the protein precipitate with the stirring rod already used. Do not stir too vigorously but only sufficiently to obtain a uniform suspension. Centrifuge again and discard the supernatant fluid. Carry out two further washings in the same way.

After finally pouring off the supernatant fluid, add 0·8 ml. of 4 N sodium carbonate and stir with the same stirring rod. Wash down the rod with a further 0·2 ml. of 4 N sodium carbonate added drop by drop down the rod. Place the tubes overnight in an oven at 85° to 95° C. to drive off water. After thorough drying, ash in a muffle furnace for two and a half hours at 600° ± 25° C. Remove, and allow to cool to room temperature. Add 2 ml. of 2 N hydrochloric acid, avoiding excessive effervescence. Mix any remaining ash with the acid and add 2 ml. of 7 N sulphuric acid and 3 ml. of water. Stir until the reaction is finished, transfer to a clean tube, and centrifuge down the insoluble residue.

For each test take two colorimeter tubes. To one add 1 ml. of dilute standard containing 0·04 μg. of iodine (as iodide), and to the other add 1 ml. of water. Pipette 3 ml. of the supernatant fluid from the test into each colorimeter tube, and add 0·5 ml. of sodium arsenite solution to each. Mix, avoiding loss of material and contamination. Place the tubes in a constant temperature bath at 39° ± 0·1° C. for ten minutes. Warm the ceric ammonium sulphate solution in the same

way, and add 1 ml. of it to each tube. It is essential that the colorimeter should be read at definite times—six and twelve minutes are used—after adding the eerie ammonium sulphate. It is suggested that this addition be made at thirty second intervals. In this way up to twelve readings can be made. If longer than thirty seconds is required to make the reading set up fewer tubes. The eerie ammonium sulphate solution must be accurately pipetted, and mixing must be rapid and complete. Use a blue filter for the colorimeter.

Plot the readings using semi-log paper against time (see Fig. 55) unless the readings are in terms of percentage light transmission. Obtain the colorimeter reading after zero time by quickly reading a reagent blank immediately after adding the eerie ammonium sulphate. The amount of iodide present in the colorimeter tube without added iodine is obtained from the formula :

Micrograms Iodine in the colorimeter tube of the unknown

$$= \frac{0{\cdot}04 \times t_2}{t_1 - t_2}$$

where $t_1$ and $t_2$ are as shown in the diagram.

Carry through a complete reagent blank omitting only the serum. Then

Plasma or serum protein-bound iodine.

$$= 100 \times \frac{7}{3} \text{ (Iodine in final aliquot} - \text{Iodine in blank).}$$

The blank was found to be 0·015 to 0·017 $\mu$g. of iodine in the colorimeter tube.

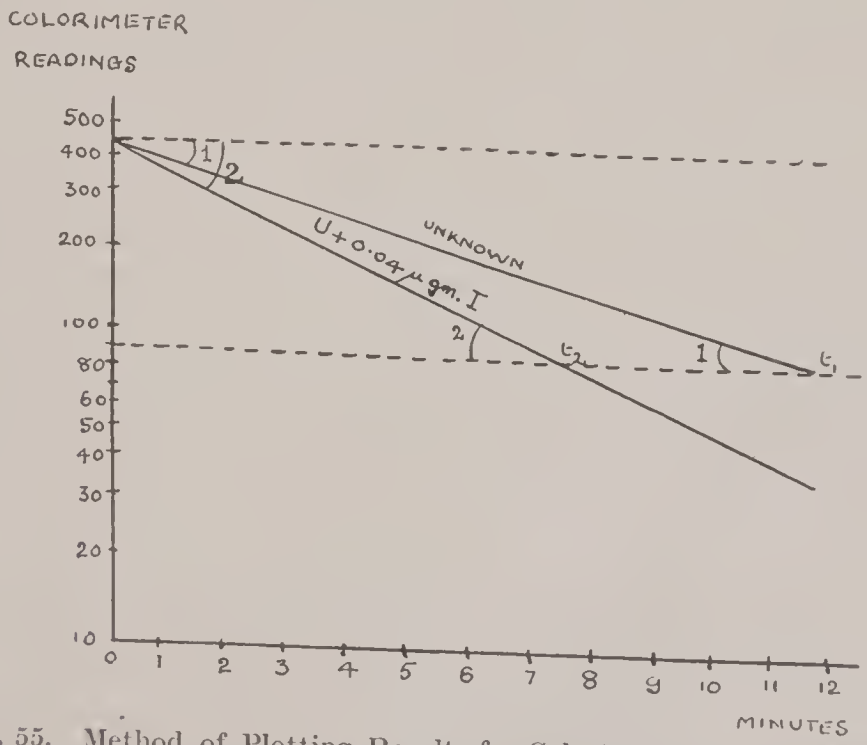


FIG. 55. Method of Plotting Results for Calculation P.B.I. (Barker, Humphrey and Soley, 1951.) Ordinates are optical density × 1000. If percentage transmission is read ordinary graph paper can be used. (*Journal of Clinical Investigation.*)

If the amount of iodine present in the test is great enough to cause complete discolorization in the second tube, repeat with less than 1 ml. of plasma and correct the calculation accordingly.

**Notes.** Glassware used must be scrupulously clean. Use of a detergent is recommended. Tincture of iodine must not be allowed in contact with the skin and iodine containing drugs must not have been taken. The latter includes the iodine-containing compounds used for X-rays.

INTERPRETATION

Different workers using different methods have reported slightly different normal ranges for the protein-bound iodine. These have, however, fallen within the range 3·5 to 8·0 $\mu$g. per 100 ml. In fact several workers have given 4 to 8 $\mu$g. per 100 ml. It is suggested (Rapport and Curtis) that the normal value should be established for the locality and for the technique used. It is unlikely that sex, season, or age influence the level of the blood iodine, though it has been found to increase during pregnancy. It is considerably affected by endogenous iodine in whatever form this is taken, so that the determination is valueless if any iodine containing compounds have been taken.

Protein-bound iodine may sometimes give a more reliable index of thyroid abnormalities than measurement of the basal metabolic rate. It is raised in hyperthyroidism, and falls on treatment whether surgical or following drugs such as thiouracil. In hypothyroidism protein-bound iodine is reduced, rising when thyroxine is given. In other diseases not involving the thyroid, abnormal values are rarely found. This may be valuable in some neuroses.

## IRON

Nearly two-thirds of the body's iron is present in hæmoglobin (334 mg. iron per 100 grams) of which about 90 per cent. is red cell hæmoglobin, the rest muscle hæmoglobin. The greater part of the remaining iron is stored, apparently combined with protein as ferritin, the liver being the tissue richest in iron. This iron is readily available when required. In addition iron is present in most tissues in enzymes such as cytochrome and catalase, which also contain hæm. Iron is thus concerned with the transport of oxygen by the blood and in cellular oxidations.

The blood iron is almost entirely present in the red cell hæmoglobin, but there is a small amount in the plasma, probably as ferric iron bound to protein (to the $\beta$-globulin fraction). This is regarded as iron which is being transported in connection with the metabolism of hæmoglobin. Accordingly determinations of serum iron are used in investigating some cases of anæmia.

### Determination of Serum Iron

Since there is only a very small amount of iron in the serum great care has to be taken to avoid any form of contamination.

Several colorimetric methods have been devised using thioeyanate (given below). ferrieyanide (Fitzpatriek and Howells, 1949). 2, 2-dipyridyl (Kitzes *et al.*, 1944 ; Watson. 1952). orthotolidine (Budtz-Olsen. 1951), o-phenanthroline (Davies *et al.*, 1952).

**Thiocyanate Method** (Brendrstrup, 1948 ; Dahl, 1948 ; Ventura and Klopper, 1951).

Hydroehloric acid is used to free the iron from its attaehment to protein, whieh is then preeipitated with triehloraeetie aeid. The colour is developed on the supernatant fluid obtained on eentrifuging, by adding nitrie aeid and thioeyanate.

**Reagents.** 1. 6 N hydroehloric acid.

2. Triehloraeetie acid, 20 per eent. aqueous solution.

3. Coneentrated nitrie aeid.

4. Potassium thioeyanate, 20 per eent. aqueous solution.

5. Ether-amyl alcohol mixture. Mix 1 volume of ether with 2 volumes of amyl aleohol.

6. Stoek standard solution. Dissolve 0·498 gram of ferrous sulphate, $FeSO_4$, $7H_2O$, in water, add 1 ml. of eoneentrated sulphurie aeid, and make up to 1 litre. This solution eontains 100 $\mu$g. per ml.

7. Standard solution for use. Dilute the stoek solution 1 in 100 to obtain a solution eontaining 1 $\mu$g. of iron per ml.

**Technique.** Add 1·5 ml. of 6 N hydroehlorie aeid to 3 ml. of serum. Shake well using a paraffined eork, and then stand one hour at 37° C. Cool, and add 3 ml. of 20 per eent. triehloraeetie aeid slowly with eontinuous shaking. Stand for a few minutes and eentrifuge. Remove the supernatant fluid, wash the deposit with 2 ml. of 5 per eent. triehloraeetic aeid, eentrifuge, and add the supernatant fluid to that already obtained. To the mixed fluids add 0·1 ml. nitric aeid and 1 ml. of 20 per eent. potassium thioeyanate. Shake, stand for ten minutes, and extraet into 5 ml. of the ether-amyl alcohol mixture, shaking well. Add a knife-point of anhydrous sodium sulphate to this extract, shake, allow to settle, and read in the photoeleetrie eolorimeter within ten minutes using a blue-green filter or transmission at 490 millimierons. The eolour of ferrie thioeyanate tends to fade easily, particularly if exposed to a bright light. Carry through a eomplete blank and a standard using 3 ml. of the dilute standard instead of serum. A standard curve ean be prepared using 3 ml. of solutions eontaining 1, 2, 3, 4 and 5 $\mu$g. of iron per ml. These correspond to 100, 200, . . . 500 $\mu$g. iron per 100 ml. of serum.

INTERPRETATION

The normal range for serum iron has been variously given. There is general agreement that it is a little higher in men than in women. The following ranges for normal may be taken,

Males, 80–175 $\mu$g. per 100 ml.
Females, 60–160 $\mu$g. per 100 ml.

(see Brendrstrup, 1948 ; Dahl, 1948 ; Watson. 1952) but both higher and lower limits have been suggested, the widest being

25–300 by Kooyman (1949). Most workers, however, have given ranges near to those above. Dahl showed that the serum iron is a little lower during menstruation than at other times.

Low figures have been obtained in the iron deficiency anæmias, and an increase in anæmias such as pernicious anæmia, in which there is reduced formation of hæmoglobin without iron deficiency.

Practically no iron is excreted in the urine in health.

**Iron-binding Capacity.**—As stated above, the iron in the plasma or serum is bound to a specific iron-binding component of the $\beta$-globulin, only part of which is saturated in normal plasma. The iron-protein complex is red, so that it is possible to measure the unsaturated iron-binding capacity, that is, the amount of iron which can still be taken up by the serum, by reading the increase in red colour produced by adding a solution of an iron salt to serum. If this is then added to the ordinary serum iron the total iron-binding capacity can be obtained. Percentage saturation can also be calculated. Such determinations have been made by Rath and Finch, 1949 ; Cartwright and Wintrobe, 1949 ; Davies *et al.*, 1952 ; and Ventura, 1952. The latter uses a solution containing 122·5 mg. of analar ferrous ammonium sulphate and 5 ml. N acetic acid in 250 ml. made up with double distilled water. This solution contains 7 $\mu$g. of iron per ml. His technique is as follows : To 1 ml. of serum diluted with 1·5 ml. of 0·85 per cent. sodium chloride add 0·1 ml. of this solution. Mix with a glass rod for two minutes, stand for a further four minutes, and read in a Beckmann spectrophotometer at 520 millimicrons using 2·5 ml. of the saline as blank. Prepare a standard curve by adding increasing amounts of iron to serum from a number of normal and anæmic persons—1, 2, 3, 4, 5 $\mu$g. amounts are satisfactory and correspond to an iron-binding capacity of 100. 200, 300 . . . $\mu$g. per 100 ml. of serum. No further increase in red colour occurs when saturation is reached.

Davies *et al.* found a total iron-binding capacity of 249–387 $\mu$g. per 100 ml. (average 304) in males, with a per cent. saturation of 25–56 (average 42) ; and of 204–429 $\mu$g. per 100 ml. (average 320) in females, with a per cent. saturation of 14–51 (average 33). Saturation is low in hypochromic anæmias—under 10 per cent. in most cases—but 100 per cent. or nearly so in pernicious anæmia and hæmochromatosis.

**Hæmosiderin.** In hæmochromatosis iron is deposited in certain tissues, such as liver and pancreas and in the skin, although serum iron is not increased. For a full account of this condition see Sheldon (1935). In the later stages pancreatic damage is sufficiently great for glycosuria and diabetes to be present (bronzed diabetes). The iron deposited gives a positive Prussian blue test with potassium ferrocyanide (equal volumes of 2 per cent. solution and 1 per cent. hydrochloric acid) and so is present in an inorganic form. A portion of skin can be taken and sections cut and stained with this reagent in suspected cases of hæmochromatosis. Iron in this form is termed hæmosiderin.

Hæmosiderin may be present in the urine in hæmochromatosis and in patients with chronic hæmoglobinuria, such as nocturnal hæmoglobinuria. This hæmosiderin is thought to be formed from hæmoglobin previously deposited in the renal tubules. It can be recognized by staining the urine deposit with the acid potassium ferrocyanide solution referred to above. Centrifuge some urine, remove the supernatant fluid, add several ml. of the ferrocyanide, mix with the deposit, stand for a few minutes, again centrifuge, and examine microscopically. Particles containing hæmosiderin stain blue.

## COPPER

Copper is required in small amounts for normal hæmoglobin synthesis. It is, however, in hepato-lenticular degeneration (Wilson's disease) that interest in copper has been aroused since it was found that there is a marked increase in the excretion of copper in this condition.

### Determination of Serum Copper

**Sodium Diethyldithiocarbamate Method** (Eden and Green, 1940 ; Ventura and King, 1951)

The copper is released from its linkage to protein by means of hydrochloric acid, the proteins precipitated by trichloracetic acid, and the copper extracted from the protein-free fluid obtained, into an amyl alcohol-ether mixture as a golden yellow-coloured complex with sodium diethyldithiocarbamate. The amount of this formed is read colorimetrically. Sodium pyrophosphate is added to prevent interference from iron.

**Reagents.** 1. N/10 hydrochloric acid.
2. 6 N hydrochloric acid.
3. Trichloracetic acid, 20 per cent. aqueous solution.
4. Sodium pyrophosphate, 6 per cent. solution.
5. Ammonia, S.G. 0·88.
6. Sodium diethyldithiocarbamate, 0·4 per cent. solution.
7. Amyl alcohol-ether mixture, containing equal volumes of each.
8. Powdered anhydrous sodium sulphate.
9. Stock standard solution. Dissolve 0·393 gram of copper sulphate ($CuSO_4$, $5H_2O$) in water, add 0·1 ml. of concentrated sulphuric acid and make up to 1 litre. This solution contains 100 $\mu$g. per ml.
10. Standard for use. Dilute the above 1 to 100 to obtain a solution containing 1 $\mu$g. per ml.

**Technique.** To 3 ml. of serum add 1 ml. of N/10 hydrochloric acid and warm in boiling water, stirring continuously, until the mixture begins to cloud. Cool. Then add 1·5 ml. of 6 N hydrochloric acid and stand for ten minutes. Add 3 ml. of 20 per cent. trichloracetic acid. Mix well, stand for a few minutes, and centrifuge. Remove the supernatant fluid and wash the precipitate with 3 ml. of 5 per cent. trichloracetic acid. Centrifuge again and combine the supernatant fluids. Add 1 ml. of 6 per cent. sodium pyrophosphate and

2 ml. of ammonia. Add 1 ml. of 0·4 per cent. sodium diethyldithiocarbamate and shake well for about two minutes with 5 ml. of the amyl alcohol-ether mixture to extract the copper. Remove the amyl alcohol layer and dry by shaking with a little powdered anhydrous sodium sulphate. Read in the photoelectric colorimeter using a violet filter or transmission at 440 millimicrons. Treat 5 ml. of standard (containing 5 $\mu$g.) in the same way as the serum, and carry through a complete blank, against which both standard and unknown are read.

**Calculation.** Micrograms copper per 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 5 \times \frac{100}{3}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 167.$$

A standard curve can be prepared by carrying through the full method using amounts of solution containing 0 (blank), 1, 2, 3, 4, 5 $\mu$g. of copper.

**Note.** Use water redistilled from an all-glass still for all solutions, and high-grade reagents.

### Determination of Copper in Urine

**Reagents.** 1. Sulphuric acid, 2 N.
2. Citric acid, 12 per cent. solution.
3. Ammonia, S.G. 0·88.
4. Thymol blue indicator.
5. Carbon tetrachloride.
6. Anhydrous sodium sulphate, powdered.
7. Sodium diethyldithiocarbamate, 0·4 per cent. solution.
8. Standard solution, as for serum.

**Technique.** Evaporate almost to fuming, about 150 to 200 ml. of 2 N sulphuric acid, which has been shaken with sodium diethyldithiocarbamate as described for deleading urine (p. 507). Add slowly 100 ml. of urine and heat carefully until organic matter is oxidized, and a colourless liquid remains. Cool, add about 20 ml. water, followed by 1 ml. of 12 per cent. citric acid, and make alkaline to thymol blue with ammonia. Cool. Add 1 ml. of the sodium diethyldithiocarbamate and 5 ml. of carbon tetrachloride. Shake vigorously for at least two minutes to extract the copper, remove the carbon tetrachloride layer, add a little sodium sulphate to remove water, and read in the colorimeter as before. Carry through a complete blank and read the unknown against this. For the standard take 5 ml. of the standard solution (containing 5 $\mu$g. of copper), add water to about 20 ml., then 1 ml. 12 per cent. citric acid, and proceed as above. Read this against a blank prepared in the same way but omitting the 5 ml. of standard.

**Calculation.** Micrograms copper per 100 ml. urine

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 5.$$

A standard curve can be prepared in the same way as described for the standard, but using 1, 2, 3, 4, 5 μg. of copper.

For urines containing increased amounts of copper, repeat, using a smaller volume of urine.

INTERPRETATION

The normal range for plasma or serum copper is within the limits 75 to 160 μg. per 100 ml. (see Cartwright, 1950, for a discussion of copper metabolism). The copper is attached to some of the globulins. Plasma copper is increased in most infections in which there is fever and an increase in the red cell sedimentation rate. In some chronic infections the range is from 160 to 250. There is also an increase during pregnancy to an average level of about 220 μg. per 100 ml. The average figure is increased a little in pernicious anæmia, in iron deficiency anæmias, and in cirrhosis of the liver.

Whereas earlier work gave relatively high values for the excretion of copper in the urine reaching 500 and 700 μg. daily, more recent work suggests this is much lower and does not exceed 70 μg. in twenty-four hours. Interest has been directed recently to the very considerable increases in urine copper excretion in Wilson's disease (hepatolenticular degeneration), in which up to and over 1 mg. may be excreted daily. An increase in plasma copper has been reported in this condition by some workers but has not been found by others. Small increases, mostly in the range 100 to 200 μg. daily, but occasionally higher, may be found in cirrhosis of the liver and in Fanconi's syndrome.

## SULPHUR

The greater part of the sulphur in the diet is present in proteins as the amino acids cystine and methionine, though there are also some non-protein organic sulphur compounds and some inorganic sulphur as sulphates. Most of the sulphur present in proteins is ultimately converted to inorganic sulphate, some of which is excreted in that form and some as ethereal sulphates, phenolic compounds such as indoxyl and skatoxyl sulphates, formed from indole and skatole produced in the intestine by the action of bacteria on protein. Important compounds which contain sulphur are insulin, glutathione, and aneurin.

The sulphur present in the blood is distributed between the different forms as follows, inorganic sulphur, average about 1 mg. per 100 ml., ethereal sulphate sulphur, 0·5 mg. per 100 ml., and neutral sulphur, 1·9 mg. per 100 ml., giving a total sulphur averaging about 3·4 mg. per 100 ml.

Determination of these sulphur compounds in blood has not found application up to the present in disease. For methods see Fiske (1921), Denis and Reed (1926–27), Wakefield (1929), and Power and Wakefield (1938); see also Peters and Van Slyke (1931). For the determination of cystine in blood, see Looney (1922), and for glutathione, see Thompson and Watson (1952).

The same types of sulphur compounds are present in the urine, in which 2 to 3 grams are excreted daily. By far the greater part is inorganic sulphate. There is about 15 per cent. as neutral sulphur, and inorganic sulphur forms about 90 per cent. of the rest leaving 10 per cent. ethereal sulphur. Ethereal sulphates such as indican are excreted in increased quantity when there is increased putrefaction in the intestine. In the Jaffé test for indican, equal volumes of urine and hydrochloric acid are mixed and a few ml. of chloroform and a few drops of calcium hypochlorite solution added. These are mixed by inverting several times. The indican is oxidized to indigo blue, which is extracted into the chloroform giving it a blue colour.

## MAGNESIUM

Determination of serum magnesium is also very rarely used. The normal range for serum is approximately 1·5 to 3 mg. per 100 ml. Most methods for determining serum magnesium remove the calcium and then precipitate the magnesium as phosphate, which is then estimated by any of the methods used for inorganic phosphorus. See, for example, Denis (1922) and Briggs (1924).

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*CHAPTER XVIII*

# CHLORIDE, SODIUM, AND POTASSIUM

Chloride, sodium and potassium ions in body fluids, because of the osmotic pressure they exert, play an important part in maintaining normal water distribution between cells, plasma and interstitial fluid. Together with bicarbonate they are also concerned with the acid-base balance in body fluids. In this chapter they will be considered together and abnormalities in volume and distribution of body water will also be discussed. Bicarbonate and changes in acid-base balance will be dealt with in the following chapter. In addition to their influence on the distribution of body water, sodium and potassium also play a part in providing the correct environment for normal muscle contraction.

## CHLORIDE

### Determination of Plasma Chloride

#### I Method of Van Slyke (1923).

See also Eisenmann (1929), Wilson and Ball (1928)

This method uses the Volhard chloride estimation, protein being oxidized by the open Carius method using a solution of silver nitrate in concentrated nitric acid. The organic matter is destroyed and the chloride precipitated at the same time. The excess silver nitrate is then titrated with standard thiocyanate.

**Reagents.** 1. N/20 solution of silver nitrate in concentrated nitric acid. Dissolve 8·495 grams of silver nitrate in about 20 ml. of water and then make up to a litre with concentrated nitric acid. The solution, which should be standardized against sodium chloride, keeps indefinitely in a brown bottle.

2. N/50 thiocyanate. Prepare at intervals by diluting a stock N/10 solution.

3. Concentrated nitric acid.

4. Five per cent. solution of ferric alum ($Fe_2(SO_4)_3$, $(NH_4)_2SO_4$, $24H_2O$).

**Technique.** Pipette 1 ml. of plasma into a 6 × 1 inch boiling tube and run in slowly, with constant shaking, 3 ml. of the silver nitrate solution. Add 2 ml. of concentrated nitric acid and heat over a bunsen flame until digestion has occurred and the solution is pale yellow in colour. If the colour is deep yellow it can be discharged by adding a few drops of saturated potassium permanganate solution and warming again. This is not necessary with plasma. The digestion usually requires only one to two minutes. When the digestion is completed, cool, and add 6 ml. of 5 per cent. ferric alum or 5 to 6 ml. of water and about 0·3 gram of powdered salt. Cool

again under running water. Then titrate with N/50 thiocyanate until a reddish brown colour persisting for ten to fifteen seconds is obtained. The end point is not easy to gauge and some experience is needed before confidence in it is acquired. Since silver thiocyanate is less soluble than silver chloride, as soon as ferric thiocyanate is formed in solution, reaction takes place between this and the precipitated silver chloride to form ferric chloride and silver thiocyanate, so that the end point fades. The speed with which this occurs is reduced if the silver chloride precipitated is coagulated into a few large pieces. Such coagulation is more likely to occur if the silver nitrate solution is added slowly drop by drop with shaking. Once technicians are familiar with the end point they have little further difficulty in obtaining accurate results.

To determine the standard add 2 ml. of nitric acid and 6 ml. of ferric alum solution to 3 ml. of the silver nitrate solution, cool, and titrate with N/50 thiocyanate. Since the silver solution is N/20, 7·5 ml. of the thiocyanate should be required. Carry out this standard titration whenever a determination is made.

**Calculation.** The difference between the two titrations gives a measure of the amount of chloride in 1 ml. of plasma in terms of N/50 solution. Since 1 ml. of N/50 sodium chloride contains 1·17 mg. per ml.

Mg. chloride (as NaCl) per 100 ml. of plasma

$$=(\text{ml. titration of standard}-\text{ml. titration of unknown})\times 1{\cdot}17\times 100$$
$$=(\text{ml. titration of standard}-\text{ml. titration of unknown})\times 117.$$

If the result is to be expressed in terms of Cl multiply by 71 instead of 117 ; for milliequivalents per litre multiply by 20.

**II Volhard Method using the Folin Wu Filtrate** (Whitehorn, 1921)

**Reagents.** 1. Silver nitrate solution. Dissolve 2·907 grams of silver nitrate in water and make up to 1 litre. One ml. is equivalent to 1 mg. of sodium chloride. Standardize against sodium chloride.

2. Concentrated nitric acid.

3. Powdered ferric alum.

4. Thiocyanate solution, ammonium or potassium. One ml. should be equivalent to 1 ml. of the silver nitrate solution. This solution can be prepared by diluting 100 ml. of N/10 solution to 585 ml. with water. Check and adjust if necessary.

**Technique.** Add 10 ml. of the silver nitrate solution with thorough stirring to 10 ml. of the Folin-Wu filtrate in an evaporating dish. Run in 5 ml. of concentrated nitric acid, stand for five minutes to allow flocculation of the silver chloride, then add about 0·3 gram of ferric alum and titrate with the thiocyanate to a reddish brown end-point which persists for ten to fifteen seconds.

**Calculation.** Mg. chloride (as sodium chloride) per 100 ml. plasma

$$=(10-\text{ml. thiocyanate used in the titration})\times 100.$$

**Note.** It is important that the sodium tungstate used for preparing the Folin-Wu filtrate should be chloride-free. Test by mixing 1

volume of the 10 per cent. sodium tungstate with 2 volumes of concentrated nitric acid and then filtering into a test tube containing some of the silver nitrate solution. Not more than a faint cloud should result. If necessary the tungstate can be purified by recrystallizing from alcohol.

### III Mercuric Nitrate Method of Schales and Schales (1941)

Plasma, or a protein-free filtrate such as that of Folin-Wu, is titrated with a mercuric nitrate solution, using diphenylcarbazone as indicator, which gives a violet-blue complex with mercuric ions. Mercuric chloride is formed, and remains in solution but is so very slightly dissociated that sufficient mercuric ions to give this colour are only present when all the chloride is titrated and excess mercuric nitrate is added.

**Reagents.** 1. Mercuric nitrate solution. Dissolve 2·9 to 3·0 gram mercuric nitrate in a few hundred ml. of water, add 20 ml. of 2 N nitric acid and make up to a litre with water. It is important that this amount of acid be strictly adhered to, otherwise the end-point will not be so sharp.

2. Diphenylcarbazone indicator. Dissolve 100 mg. in 100 ml. of 95 per cent. alcohol. Keep in the dark in the refrigerator. The solution fades in light within a few days and cannot be used. Prepare freshly once a month.

3. Standard chloride solution. Dissolve 585 mg. of sodium chloride, dried at 120° C., in water and make up to 1 litre.

**Technique.** Use 2 ml. of Folin-Wu filtrate or 0·2 ml. of plasma and 1·8 ml. of water. Add 0·06 ml. of the indicator and titrate with the mercuric nitrate using a microburette calibrated to 0·01 ml. One ml. should equal 100 drops. The end-point is better with protein-free solutions which give an intense violet-blue colour when the first drop of excess mercuric nitrate solution is added. When the titration is done directly on plasma the colour at first is salmon-red, changing to deep violet when the titration is begun then becoming pale yellow or colourless. A sharp change to pale violet then denotes the end-point. Carry out the titration on 2 ml. of the standard chloride solution.

**Calculation.** Mg. chloride per 100 ml. plasma

$$= \frac{\text{ml. mercuric nitrate solution required for the unknown}}{\text{ml. mercuric nitrate solution required for the standard}} \times 585.$$

### IV Determination by the Silver Iodate Method (King and Bain, 1951)

Several workers have used the reaction of silver iodate with sodium chloride in the protein-free filtrate from plasma to form a precipitate of silver chloride leaving sodium iodate in solution. Sendroy (1937, 1939, 1942), and Van Slyke and Hiller (1947) shake with solid silver iodate, filtering off the precipitated silver chloride and excess iodate. King *et al.*, (1937) used an ammoniacal solution of silver iodate, and filtered the silver chloride and excess iodate after precipitating the latter by adding acid. In both cases the iodate in solution, which is

equivalent to the chloride present in the plasma used, is determined by adding potassium iodide and titrating the iodine liberated, with thiosulphate. King and Bain (1951) have further simplified King's original method by precipitating the proteins, silver chloride, and excess silver iodate together.

**Reagents.** 1. Ammoniacal silver iodate solution. Prepare a stock solution by dissolving 1·8 grams of silver iodate in 100 ml. of normal ammonia solution. From this prepare freshly the solution for use in the test. Mix well equal volumes of the stock solution and normal sulphuric acid in a centrifuge tube, say 5 ml. of each, and centrifuge off the precipitated silver iodate. Pour off the supernatant fluid, dissolve the precipitate in a volume of 0·3 N ammonia equal to the volume of stock solution used. This removes any soluble iodate from the silver iodate.

To prepare silver iodate, mix equal volumes of solutions containing 4·28 grams of potassium iodate per 100 ml. and 3·4 grams of silver nitrate per 100 ml. Filter, wash the precipitate well with distilled water, dry in a vacuum desiccator and keep in the dark.

2. Normal ammonia solution. Dilute 57 ml. of concentrated ammonia, specific gravity 0·88, to 1 litre with water.

3. Ammonia, 0·3 N solution. Dilute 17 ml. of S.G. 0·88 ammonia, to 1 litre.

4. Tungstate-phosphoric acid reagent, Dissolve 4·2 grams of sodium tungstate ($Na_2WO_4$, $2H_2O$) in 1 litre of phosphoric acid (0·15 M, prepared by diluting 10 ml. of the concentrated acid, specific gravity 1·72, to 1 litre with water).

5. Potassium iodide, 2 per cent. solution. Dissolve 2 grams in water and make up to 100 ml.

6. N/200 sodium thiosulphate.

**Technique.** Add 0·5 ml. of ammoniacal silver iodate solution to 0·2 ml. of plasma, mix and add 3·3 ml. of tungstic-phosphoric acid reagent. Shake well and filter, using a 7 cm. No. 42 Whatman paper. Treat 1·0 ml. of the filtrate with 1·0 ml. of 2 per cent. potassium iodide and titrate with N/200 thiosulphate with starch as indicator.

**Calculation.** Mg. chloride (as sodium chloride) per 100 ml. plasma

$$= \text{ml. titration} \times 97{\cdot}5.$$

**Note.** If whole blood is taken, use double the amount of sodium tungstate in the tungstate-phosphoric acid reagent.

### Choice of Blood for Chloride Estimation

Plasma separated immediately from blood taken under liquid paraffin is best used. Plasma is used because of the very unequal distribution of chloride between cells and plasma. Since there is about twice as much chloride in plasma as in the cells an appreciable alteration in the red cell count can mask the changes in plasma chloride with which we are mainly concerned in pathological states. Thus in anæmia, whole blood chloride rises because of the greater proportion of plasma whilst the opposite tendency may be manifest in polycythæmia. However, if the red cell count is normal

whole blood can be used. If plasma is used, precautions are usually taken to avoid chloride shift due to loss of carbon dioxide from whole blood when this is allowed to stand exposed to the air (see p. 6). The blood is thus collected under oil and the plasma separated as soon as possible. It can then be kept without further alteration.

**Choice of Method.** The method of Van Slyke is simple and quick but the end-point is not so good as that in the methods of King and Bain and of Schales and Schales so that one of these may be preferred.

**Normal Values.** Expressed in terms of sodium chloride these are :

| | |
|---|---|
| Whole blood | 450 to 510 mg. per 100 ml. ; 77 to 87 milli-equivalents per litre. |
| Plasma | 560 to 620 mg. per 100 ml. ; 95 to 106 milli-equivalents per litre. |

Variations in chloride will be considered later, when sodium is being discussed.

### Determination of Urine Chloride

The **method of Van Slyke** given for blood can be used for urine. Take 1 ml. of urine instead of 1 ml. of plasma. If the chloride content exceeds about 800 mg. per 100 ml. take 0·5 ml. of urine, and make the necessary alteration in the calculation. This method is very convenient on account of its simplicity and the end-point is better than with blood.

### Approximate Method of Fantus (Fantus, 1936)

Marriott (1947) uses an approximate method devised by Fantus which is very simple and can be carried out where laboratory facilities are not available.

**Reagents.** 1. Silver nitrate, 2·9 per cent. solution.
2. Potassium chromate, 20 per cent. solution.
3. Distilled water.

**Technique.** Place 10 drops of the urine into a test tube from a pipette. Wash the pipette out with distilled water and then add 1 drop of the chromate. Again wash out the pipette with water. Then add the silver nitrate drop by drop counting the number of drops required to give a brick-red precipitate.

**Calculation.** Number of drops of silver nitrate required

= Grams sodium chloride per litre of urine.

**Note.** Some criticism of this test has been made on the ground that it is not entirely reliable (see Taylor, 1951). Certainly the Volhard method of Van Slyke and Sendroy is almost as quick and completely reliable so that it should be done when there is a laboratory.

**Urine chloride** is greatly influenced by the amount of salt taken in

the diet. A usual amount is about 10 grams daily so that the average excretion is about this amount, ranging from 7 to 15 grams. On low intakes of salt less is excreted, and when the plasma level falls below the lower limits of normal, that is below 560 mg. per 100 ml., excretion of chloride ceases almost completely. A person on a normal intake who takes an increased amount excretes this in twenty-four to forty-eight hours, but one with a previously low intake of salt does not do so for several days. In normal persons chloride can be excreted up to a concentration of about 2 per cent.

## SODIUM

### Determination of Serum Sodium

Sodium has been most frequently determined by precipitation as sodium zinc uranyl acetate, which, although it dissolves in water, is sufficiently insoluble in alcohol. Various methods have been used for the colorimetric determination of the amount of precipitate obtained. One of the commonest is to treat with potassium ferrocyanide when a red colour due to uranyl ferrocyanide is produced (McCance and Shipp, 1931 ; Weinbach, 1935 ; Noyons, 1939 ; King *et al.*, 1942). Butterworth (1951) uses the orange colour given with sodium salicylate, whilst Stone and Goldzieher (1949) obtain a reddish-yellow colour with hydrogen peroxide in presence of alkali. Some methods stand overnight to obtain precipitation, a disadvantage since results are usually required quickly if they are to be of value. Butterworth uses two hours, Stone and Goldzieher only twenty minutes.

The amount of sodium zinc uranyl acetate formed has also been determined gravimetrically (see Harrison, 1947). Further, instead of determining the amount of precipitate some workers have measured the loss of colour of the reagent used for precipitating the sodium. Fowweather and Anderson (1948) use zinc uranyl acetate, Trinder (1951) magnesium uranyl acetate in this way.

Sodium has also been precipitated as the pyroantimonate, the amount of which can be determined iodometrically.

Recently the flame photometer has been applied to the determination of sodium in serum and urine and has made it possible to carry out rapidly large numbers of estimations.

### I. Zinc Uranyl Acetate Method (Stone and Goldzieher, 1949 ; Butterworth, 1951)

After removal of the proteins, the sodium is precipitated as zinc sodium uranyl acetate. The precipitate is washed and treated with sodium salicylate, and the resulting orange colour read in the colorimeter.

**Reagents.** 1. Trichloracetic acid, 10 per cent. (w/v) solution.

2. Uranyl zinc acetate. Add 14 ml. of glacial acetic acid to 750 ml. of distilled water and bring almost to the boil. Add 77 grams uranyl acetate. Add 231 grams of zinc acetate, divided into five or six portions, stirring frequently. Finally add 7 ml. glacial acetic acid,

cool to room temperature, and make up to 1 litre. Then add 200 ml. 95 per cent. ethyl alcohol, place in the refrigerator overnight and, filter.

3. Triple salt. To 50 ml. of the uranyl zinc acetate add about 100 ml. of 50 per cent. alcohol saturated with sodium chloride. Stand. Filter by suction through a sintered glass filter, wash thoroughly with alcohol, then finally with ether. Dry in a desiccator. Dissolve 0·401 gram in water and make up to 200 ml. One ml. is equivalent to 0·030 mg. sodium.

4. Wash reagent. Absolute alcohol saturated with magnesium acetate. Filter before use.

5. Sodium salicylate. 1 per cent. solution made up accurately.

6. Uranyl acetate, 0·2 per cent. solution.

7. Standard sodium chloride solution A (1·8 mg. sodium per ml.). Dissolve 0·458 gram of pure dry salt in water and make up to 100 ml.

8. Standard sodium chloride solution B (0·18 mg. sodium per ml.). Dilute the standard A, 1 in 10.

**Technique.** Add 0·3 ml. of serum drop by drop to 4·7 ml. of 10 per cent. trichloracetic acid. Shake well and centrifuge. Pipette 0·5 ml. of the supernatant fluid (= 0·03 ml. serum) into a centrifuge tube and add 3 ml. of uranyl zinc acetate reagent. Mix well by rotating. Stand for twenty to thirty minutes, mixing several times by rotation during that time. Centrifuge, decant the supernatant fluid, and allow the tube to drain on filter paper for one minute. Add 5 ml. of wash reagent, washing down the side of the tube. Mix by twirling with a fine glass rod, centrifuge, decant and drain as before. Dissolve the precipitate in 5 ml. of distilled water, and add 5 ml. of 1 per cent. sodium salicylate solution. Mix and read in the colorimeter with a blue filter or transmission at 460 millimicrons and using 0·2 per cent. uranyl acetate as blank. At the same time put through a determination using 0·5 ml. of the standard solution B instead of supernatant fluid. Then Mg. sodium per 100 ml.

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}09 \times \frac{100}{0{\cdot}03}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 300.$$

Alternatively, take 3 ml. of triple salt solution and add 2 ml. of water and 5 ml. of sodium salicylate.

A standard curve can be prepared as follows :

| Mg. sodium per 100 ml. serum | 0 | 100 | 200 | 300 | 400 | 500 |
|---|---|---|---|---|---|---|
| Ml. triple salt solution (1 ml. = 0·03 mg. Na) | 0 | 1·0 | 2·0 | 3·0 | 4·0 | 5·0 |
| Ml. water | 5·0 | 4·0 | 3·0 | 2·0 | 1·0 | 0 |
| | Add 5 ml. sodium salicylate solution. | | | | | |

Check the 300 mg. per 100 ml. point against the standard sodium chloride B put through as above.

### II. Magnesium Uranyl Acetate Method of Trinder (1951)

The sodium and the proteins are precipitated together by a magnesium uranyl acetate reagent, are removed by centrifuging and the sodium determined by measuring the loss in concentration of the reagent solution.

**Reagents.** 1. Magnesium uranyl acetate reagent. Mix 8 grams of uranyl acetate ($UO_2(C_2H_3O_2)_2$, $2H_2O$), 30 grams of magnesium acetate ($Mg(C_2H_3O_2)_2$, $4H_2O$), and 30 ml. of glacial acetic acid with 150 ml. of water and heat to dissolve. Then boil for two minutes, cool, dilute to 200 ml. with water and make up to a litre with absolute ethyl alcohol. Place in a brown bottle and add 1 ml. of 1 per cent. sodium chloride. Mix and stand for several days until the precipitated triple acetate settles out. Use the supernatant solution.

2. Potassium ferrocyanide ($K_4Fe(CN)_6$, $3H_2O$), 10 per cent. solution. Keep in a brown bottle.

3. Acetic acid, 1 per cent. (v/v).

4. Standard sodium solution (500 mg. per 100 ml.). Dissolve 1·271 grams of dry sodium chloride in water and make up to 100 ml.

**Technique.** Pipette 5 ml. of the magnesium uranyl acetate reagent into each of two glass-stoppered centrifuge tubes ($4 \times \frac{5}{8}$ inch). To one add 0·1 ml. of serum from a pipette calibrated to contain and agitate the solution vigorously by blowing through the pipette. To the other, used as blank, add 0·1 ml. of water. Stopper and stand for five minutes to allow formation of the precipitate of triple acetate. Shake the tubes vigorously for thirty seconds and centrifuge. Measure 2 ml. of the supernatant fluids into 100 ml. volumetric flasks, add about 80 ml. of 1 per cent. acetic acid, 4 ml. of 10 per cent. potassium ferrocyanide, and make to the mark with 1 per cent. acetic acid. Mix, and read within the next ten minutes using the unknown, in the case of a direct reading photoelectric colorimeter, to set the instrument to full scale deflection, and then reading the blank. Use a blue filter or transmission at 480 millimicrons. Use of 10-mm. cells is recommended.

In order to obtain a standard curve for the colorimeter, prepare from the standard solution given above, a series of solutions containing 100, 200, 300, 400 and 500 mg. of sodium per 100 ml. and treat 0·1 ml. of each of these in the same way as the unknown. When carrying out tests put up 0·1 ml. of a standard solution containing 300 mg. sodium per 100 ml.

### III. The Potassium Pyroantimonate Method (Kramer and Gittelmann, 1924–25 ; Rourke, 1928)

Sodium is precipitated as the pyroantimonate directly from the serum and the amount of antimony in the precipitate is determined iodometrically by titration with sodium thiosulphate after addition of potassium iodide.

**Reagents.** 1. Potassium pyroantimonate reagent. Heat 500 ml. of distilled water to boiling in a pyrex flask. Remove the bunsen burner and after a few moments add 5 grams of potassium pyroantimonate. Replace the burner and boil for three to five minutes.

cool immediately under running water and when cold add 15 ml. of 10 per cent. potassium hydroxide (alcohol washed). Filter through ash-free filter paper into a paraffin-lined bottle. If there is a deposit after filtering, allow to settle for twenty-four hours and use the supernatant fluid. This reagent has been said to keep for one to two months. It is better to prepare the reagent freshly every week.

The reagent can be tested for the presence of sodium and to show that none of the potassium pyroantimonate is precipitated by the addition of alcohol as used in the test, by adding 2 ml. of distilled water and 3 ml. of 95 per cent. alcohol to 10 ml. of the reagent. The reagent as used should have a *p*H of approximately 9 and should contain between 31 and 37 mg. of antimony. Because of the variable purity of the salt it is advisable to test as follows. Place 2 ml. of the reagent into a 100-ml. pyrex flask, add 15 ml. distilled water, 5 ml. concentrated hydrochloric acid, and 2 ml. 20 per cent. potassium iodide and titrate with N/20 thiosulphate. 4·7 ml. of this should be required.

2. Ethyl alcohol, 95 per cent. (v/v from alcohol redistilled over potassium hydroxide).

3. Ethyl alcohol, 30 per cent. (v/v).

4. N/20 sodium thiosulphate.

5. Potassium iodide, 20 per cent. solution. Prepare a small amount freshly before use.

6. Concentrated hydrochloric acid.

7. Starch, 1 per cent. solution.

**Technique.** Pipette 0·5 ml. of the serum (or, if desired, the ash of an equal amount of serum dissolved in normal hydrochloric acid and made alkaline with 4 drops of 1·8 N potassium hydroxide (alcohol washed) ) in a tapered centrifuge tube. Add 5 ml. of the pyroantimonate reagent, cool to 10° C. and then add exactly 1·5 ml. of the 95 per cent. alcohol, also cooled to 10° C., drop by drop, whilst stirring with a rubber-tipped glass rod. This addition should take at least thirty to forty-five seconds. Stopper the tube and allow to stand for forty-five to sixty minutes. Centrifuge. Without disturbing the precipitate, remove as much as possible of the supernatant fluid. Add 5 ml. of 30 per cent. alcohol, mix with the supernatant fluid remaining and centrifuge. Remove the supernatant fluid. Add 5 ml. of concentrated hydrochloric acid to the precipitate and dissolve by mixing, using the glass rod already used. Transfer to a 100 ml. Pyrex beaker, washing in three times with 5 ml. of distilled water. Add 1 ml. of 20 per cent. potassium iodide and titrate with N/20 thiosulphate using a 5 ml. microburette graduated to 0·02 ml. Add a drop or two of starch solution when the colour of the iodine has almost disappeared.

**Calculation.** Each ml. of N/20 thiosulphate is equivalent to 0·575 mg. of sodium. Hence

Mg. of sodium per 100 ml. of serum

$$= \text{ml. of thiosulphate required in the titration} \times 0{\cdot}575 \times \frac{100}{0{\cdot}5}$$

$$= \text{ml. of thiosulphate required} \times 115.$$

**Note.** The precipitate has the formula $Na_2H_2Sb_2O_7$ from which in presence of hydrochloric acid pentavalent antimony is liberated and reacts with the iodide to form trivalent antimony and liberates iodine.

### IV. Determination by Means of the Flame Photometer

In recent years the development of the flame photometer has made possible the rapid and accurate determination of sodium and potassium so that large numbers of estimations can be done in a short time. In this instrument the solution under test is passed under carefully controlled conditions as a very fine spray in the air supply to a burner. The light from the flame is passed through suitable filters on to a selenium cell or other photosensitive element, and the amount of current thus produced is measured. This naturally varies with the concentration of sodium, for example, in the solution which is being tested. Using solutions of known sodium content, a calibration curve can be constructed for the instrument and this used for reading the sodium content of the fluids examined. In the United States flame photometers have been on the market for some years. Recently in this country an instrument has been produced by Evans Electroselenium. Alternatively the components can be bought and assembled, and a number of home-made instruments are in use. The reader is referred to the papers of Barnes, Richardson, Berry and Hood (1945) and of Berry *et al.*, (1946) where the principles are discussed and an American type of instrument described ; also to the papers of Domingo and Klyne (1949), Spencer (1950), Brealey and Ross (1951), Mitchell (1950), Gilbert, Hawes and Beckman (1950), Hald (1947, 1951).

Several gases have been used for the flame. These include acetylene, coal gas, propane and butane. Both the gas pressure and air pressure have to be carefully regulated so as to maintain a constant steady flame, which should be blue in colour and have no yellow streaks. Whilst ordinary coal gas is more variable in composition than the others it has been satisfactorily used. The temperature of these flames differs considerably. This is important in regard to the extent to which one element can increase the excitation of another, an effect which will be referred to later.

The spray is formed by passing compressed air through an atomizer, into which the liquid which is being tested is drawn either by suction or gravity, and then enters the burner as its air supply. The pressure used is usually about 10 to 15 lb. per square inch. The atomizer is perhaps the most important part of the apparatus. It is essential that a steady fine spray of particles of uniform size should be produced if there is to be a constant emission of light. The atomizer should not clog up readily and should be easily cleaned. Domingo and Klyne used a glass atomizer, which can still be purchased, but most instruments have one made of metal which is less fragile. A glass atomizer is also not so easily reproduced. There is usually sufficient space in the atomizer for the coarser particles to fall out and to drain off.

The light produced is first passed through a lens to focus it and then through suitable filters before it falls on to the photosensitive element. For determining sodium an orange filter and a blue filter can be used. These together make it possible to read the intensity of the D line (589 millimierons). For potassium a deep red filter is used. Suitable filters are : orange, OY 1 ; blue, Ilford 803 ; deep red, Ilford, 609. For the photosensitive element, Domingo and Klyne used an ordinary selenium cell for sodium, and a gas-filled cæsium cathode cell for potassium. With phototubes, however, it is usually necessary to amplify the current produced. In the EEL flame photometer an ordinary selenium cell is used for determining both sodium and potassium. A mirror collects the light which is then focussed on to the cell. In this way sufficient current is produced even for potassium at comparatively high dilution. Selenium cells are also available which are sensitive well into the infra-red, and these can be used with the above filters for determining both sodium and potassium. An example of such a cell is the megatron infra-red cell of Megatron Limited. When a single cell is used, a device is included by which the filters can be changed so that either sodium or potassium can be determined. The amount of current produced is read on a suitable galvanometer.

Fig. 56. Flame Photometer. EEL model (Evans Electroselenium).

The most satisfactory dilution to use should be established experimentally. For sodium this may be 1 in 100 or higher, for potassium 1 in 50. In determining the amount of any element present, possible interference of other elements also present has to be taken into account. In serum there is approximately sixteen times as much sodium as potassium so that while the latter has a negligible effect on the sodium, the sodium present may increase the amount of light produced by the potassium. This effect is greater, the higher the temperature, so that it is less with a propane flame than with coal gas, and greatest of all with acetylene. This effect should not be confused with the interference due to the lines of one element with those of another. The interference due to sodium on the determination of potassium in serum, which has a relatively constant amount of sodium, can be discounted by introducing the same amount of sodium into the standards as is present in the serum. However, in some instruments, for example the EEL flame photometer described below, the effect is said not to be great enough to introduce an appreciable error, and can be ignored.

So far we have been considering the simplest type of instrument using one cell when a determination is made—*direct flame photometry*—in which the galvanometer reading is proportional to the concentration of substance present. As an example we have the EEL flame photometer shown in Fig. 56 and diagrammatically in Fig. 57. Air from a compressed air cylinder fitted with suitable reducing valves or from an air compressor, is introduced into the all-metal atomizer (1) through a control valve (2) at a pressure indicated on a gauge (3) mounted at the front of the instrument. This stream of air draws liquid from the sample being tested in beaker (4) up the inlet tube (5) and atomizes it to a fine spray. The atomizer clips into a plug (6) at one end of the spray chamber (7) in which the larger droplets fall from the air stream and flow to waste through the drainage tube (8). Gas (ordinary coal gas or calor gas) is introduced into the spray chamber through the inlet tube (9) which is connected by synthetic rubber tubing to the automatic gas pressure stabilizer (10) and to a control valve (11). The gas-air mixture burns in a broad, flat flame, and the hot gases pass up a well ventilated chimney (12). Gauze in the burner tube prevents serious firing back of the flame. The light emitted by the flame is collected by a reflector (13) and focussed by a lens (14) through the interchangeable optical filters (15) on to an EEL barrier layer selenium photocell (16). The current produced is taken through a calibrated potentiometer (17) to a galvanometer unit (18). A glass window (19) is placed between the lens and the filter for cooling purposes. The apparatus can easily be dismantled for cleaning. The dilutions of serum used are 1 in 500 for sodium and 1 in 50 for potassium. Full instructions are supplied with the instrument.

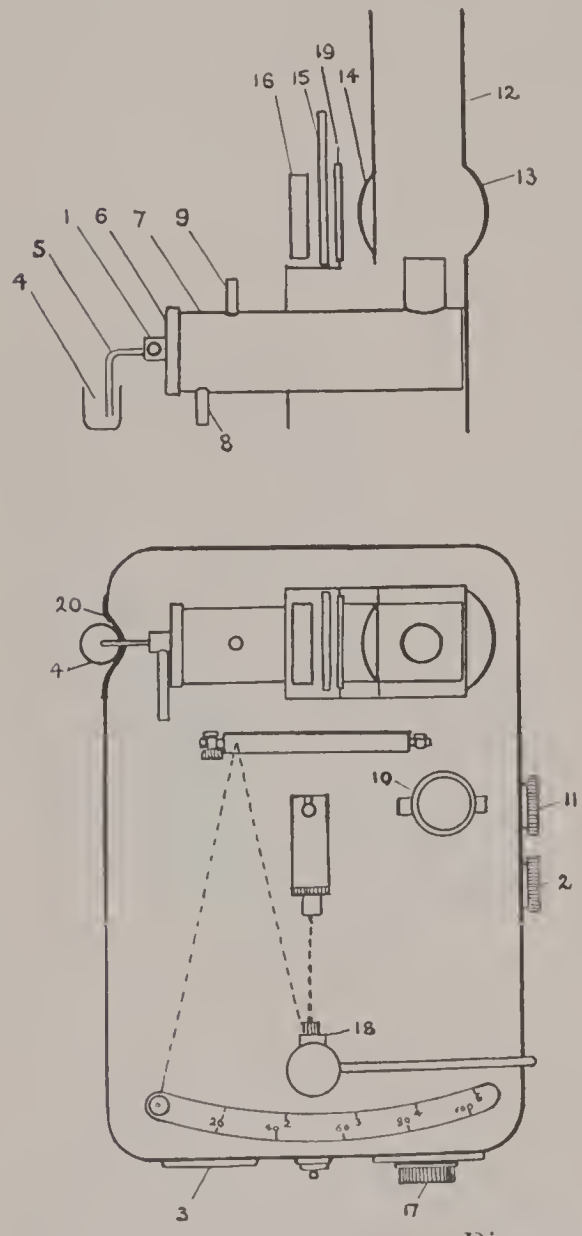


FIG. 57. Flame Photometer. Diagram of EEL model.

A different method was introduced by Barnes *et al.*, (1945). This uses an *internal standard*. A fixed amount of an element not present

in the specimen being examined is introduced so that the ratio of the intensity of the light emitted by the substance which is being determined can be compared with that from the substance added. In this way several sources of error in the direct reading instrument can be overcome. Thus fluctuations due to variations in the atomizer and flame are almost eliminated since they affect both substances equally. Lithium has been used for this purpose since it is not present in blood and is closely related to sodium and potassium. In instruments using this principle, two photocells are used arranged as two arms of a Wheatstone bridge. The reading on the scale thus corresponds to the point at which no current is passing through the galvanometer. An internal standard type of flame photometer is described by Spencer (1950).

**Normal Values for Sodium.** The sodium of the blood is almost entirely in the plasma. The normal range for serum sodium is 310–345 mg. per 100 ml., or 136–150 milliequivalents per litre. Whole blood sodium averages about 190 mg. per 100 ml.

### INTERPRETATION OF CHANGES IN SODIUM AND CHLORIDE

**Loss of chloride** may occur in the urine, from the alimentary tract in the vomit or in diarrhœa, and in the sweat.

1. **Loss from the Alimentary Tract.** Because of the considerable amount of chloride present in the gastric secretion, prolonged vomiting from whatever cause leads to a reduced content of chloride in the plasma. Thus in pyloric obstruction large amounts of gastric contents may be lost in this way and very low plasma chloride levels result. When the gastric contents contain a high concentration of hydrochloric acid as in pyloric stenosis due to duodenal ulcer, there is a much greater loss of chloride than of sodium, which is retained in the plasma as sodium bicarbonate. In such cases the plasma chloride may fall to below 300 mg. per 100 ml., that is to approximately 50 per cent. of the normal level, whereas sodium does not fall to such an extent and does not go below about 250 mg. per 100 ml. When there is vomiting in gastric disease in which there is achlorhydria, loss of chloride more closely parallels loss of sodium and the plasma chloride does not reach such low figures.

Vomiting may occur in a number of other conditions, for example, uræmic and diabetic coma, toxæmia of pregnancy, and contribute to the low plasma chloride levels found.

Severe prolonged diarrhœa may also lead to loss of sufficient sodium chloride to reduce the plasma chloride considerably. Since bicarbonate is present in the secretions into the small intestine, the fall in serum sodium in diarrhœa may exceed that of chloride so that plasma bicarbonate tends to be reduced a little, in contrast to the increase which often accompanies prolonged vomiting. Severe diarrhœa is found more commonly in young children in this country, but occurs in adults in such conditions as ulcerative colitis and is found in tropical diseases such as cholera. In ulcerative colitis, for example, plasma chloride may fall well below 400 mg. per 100 ml.

2. **Loss in the Urine.** While chloride excretion practically ceases in normal persons when the plasma chloride falls below the lower limits of normal, in some conditions chloride excretion continues even when there are subnormal levels of plasma chloride. Thus some chloride is excreted in the urine in the polyuria of advanced chronic nephritis and diabetes, even though the plasma chloride is appreciably below the lowest normal values. The most interesting condition in which chloride continues to be excreted at low plasma chloride levels is Addison's disease, adrenal cortical insufficiency, in which there is a deficiency of the hormone which controls the excretion of salt and the distribution of sodium and potassium in the body fluids. Excretion of salt continues even when the plasma chloride is well below 500 mg. per 100 ml. It is, however, only in the crises of the disease, or when these are approaching, that such considerable abnormalities develop. Then the plasma chloride may fall to below 400 mg. per 100 ml. whilst the serum sodium is reduced towards 250 mg. per 100 ml. At other times these are normal, or nearly so.

Tests have been devised to aid in the diagnosis of Addison's disease, which are based on these and other abnormalities associated with them. One is the *test of Cutler, Power, and Wilder* (1938) in which urine chloride is determined after the patient has been placed on a low sodium chloride and high potassium diet. Cutler gives a standard diet which contains 0·95 gram of chloride, 0·59 gram of sodium, and 4·1 gram of potassium for three days. Salt-free bread and butter are used, and salt is not used in cooking. Additional potassium in the form of potassium citrate (one-third of a gram per kilogram on the afternoon of the first day and on the morning of the second day) is given. During the second day 40 ml. of fluid per kilogram is given, and 20 ml. per kilogram before 11 a.m. on the morning of the third day. Urine is collected over a four-hour period from 8 a.m. to 12 noon, on the morning of the third day, that is forty-eight to fifty-two hours after beginning the test. Urine chloride is determined. Normal persons were found to have a range from 17 to 141 mg. per 100 ml. (as NaCl), with a mean of 55, while in Addison's disease the range was 229 to 356 mg. per 100 ml., average 293. The ranges for sodium were 6 to 85 mg. per 100 ml. and 165 to 282 mg. per 100 ml. respectively. Whether the dividing line is always so definite is doubtful. However, a value of more than 230 strongly suggests a diagnosis of Addison's disease, while one of less than 140 should exclude it.

The test is a provocative one and is not without danger since it may precipitate a crisis of the disease. It has been recommended that at the end of the test intravenous glucose and salt should be given with some adrenal-cortical extract. Because of this risk the test has been largely discontinued and in its place the Kepler, Robinson and Power test used. This needs no salt restriction and is not likely to produce a crisis. If adrenal extracts or synthetic products are being given, they must be stopped two to three days before doing the test, but since it is mainly used for diagnosis, this is not likely to be necessary.

**The Water Excretion Test of Kepler, Power and Robinson (1941)**

This test, which is in two parts, is based respectively on two facts. In the first place patients having Addison's disease do not usually experience diuresis after the rapid intake of a considerable amount of water. They do excrete the excess of water eventually but the time required is much longer than with normal persons, that is, in most cases of Addison's disease the kidneys continue to excrete a fairly concentrated urine even after the patient has drunk a large volume of water. In the second place there is a tendency for excessive amounts of sodium chloride to be excreted but for urea to be retained. The test is as follows.

**Part I.** (Based on the volume of urine.) On the day preceding that on which the test is to be done the patient eats three ordinary meals with fluids as desired but does not take any extra salt. No further fluid or food is allowed after 6 p.m. At 10.30 p.m. the patient empties the bladder, the urine being discarded. From then onwards all the urine is collected and added to that passed at 7.30 a.m., when the bladder is again emptied. The volume of this combined urine, excreted by the kidneys between 10.30 p.m. and 7.30 a.m., and called the night urine, is measured and the specimen kept for possible chemical analysis later.

No breakfast is allowed.

A specimen of blood for estimation of blood urea and plasma chloride is taken between 8 and 8.30 a.m. and kept for possible use if part II of the test has to be proceeded with.

The bladder is again emptied at 8.30 a.m. and then within the next forty-five minutes a volume of water, corresponding to 20 ml. per kilogram of body weight (9 ml. per lb.), is drunk. The patient empties the bladder at 9.30, 10.30, 11.30 a.m., and 12.30 p.m., being kept at rest in bed except when passing the urine specimens. These are kept separately, their volumes measured and that of the largest one noted. The patient with Addison's disease may not be able to pass urine hourly. Then the volume excreted per hour may be calculated, though often such calculation is not necessary because of the low output for the whole of the four-hour period.

The criteria to be used in interpreting the results are : (1) The test is negative if the volume of the largest of the hourly morning urine specimens is greater than the volume of the night urine. The authors state that they met no exception to this. (2) Addison's disease may or may not be present if the volume of the largest hourly morning specimen is less than the volume of the night urine. In this event the second part of the test is carried out.

**Part II.** (Based on the chemistry of the blood and urine.) Urea and chloride estimations are carried out on the night urine and on the plasma from the fasting blood collected as described. From these and the volumes of the largest hourly morning urine specimen and of the night urine a factor is calculated as follows :

$$\text{Factor} = \frac{\text{Urea in urine (mg. per 100 ml.)}}{\text{Urea in plasma (mg. per 100 ml.)}} \times \frac{\text{Chlorides in plasma}}{\text{Chlorides in urine}}$$

$$\frac{\text{(mg. per 100 ml.)}}{\text{(mg. per 100 ml.)}} \times \frac{\text{Volume of largest hourly morning urine in ml.}}{\text{Volume of the night urine in ml.}}$$

The criteria to be used in interpreting this are :

(1) If the factor is greater than 25, the patient has probably not got Addison's disease.

(2) If the factor is less than 25 the patient probably has Addison's disease, provided the presence of renal failure can be excluded. Low values are also obtained in secondary hypoadrenalism due to hypopituitarism.

It is suggested that when the result of Part II of the test is equivocal or if it is negative when there is strong clinical evidence in favour of Addison's disease, the Cutler, Power and Wilder test referred to above may be carried out and proceeded with immediately.

Kepler, Power and Robinson obtained low factors in cases of nephritis, diabetes insipidus and in a case of dehydration and fever. The question of Addison's disease only arose in two of these cases and it was excluded by the Cutler, Power and Wilder test. See also Levy, Power, and Kepler (1946).

The author has used this test for some years and found it a useful procedure. No untoward effects were noted in any case.

**3. Loss of Chloride in the Sweat.** Considerable quantities of sodium chloride may be lost in the sweat. In this connection it is important to remember that there are two kinds of sweat, insensible and sensible. The former evaporates continuously from the body under all conditions in an amount averaging about 600 ml. daily. It contains little chloride. Sensible sweat secreted when the body temperature rises, may contain from 100 to 500 mg. of sodium chloride per 100 ml., averaging about 250 mg. per cent. in Europeans. Whilst little salt is lost ordinarily in this way considerable quantities may be lost when there is heavy perspiration. Whether or not there is a fall in plasma chloride depends on whether or not water is drunk in amounts sufficient to make good the loss of water. Since sweat is usually hypotonic compared with plasma, if there is no replenishment of fluid, loss of water may be greater than the loss of salt so that while plasma volume tends to fall, plasma chloride tends to increase. However, if adequate water is drunk, plasma chloride falls and similar symptoms and changes in blood constituents are found as in the previously mentioned cases of low plasma chloride. Since this condition may be found in workers who perspire freely and because muscular spasms are one of the symptoms, this has been termed " miner's " cramp, though it may occur in any person who sweats freely and only makes good the water lost.

**Reduced Chloride in some Infectious Diseases.** Although the explanation is far from clear, low plasma chloride levels are observed in a number of acute infections. Thus in meningitis the low cerebrospinal fluid chloride parallels a low plasma chloride. There is also a reduced level of chloride in both plasma and urine in lobar pneumonia.

We have already discussed the plasma chloride level in **renal disease** (p. 124) and seen that very variable findings are the rule. The factors making for reduced chloride level are vomiting, the polyuria and the formation of œdema fluid and also the effect of a possibly reduced intake of salt. Consequently, low plasma chloride levels frequently tend to be found in the later stages of chronic glomerulonephritis but are less frequently seen in other stages and types of renal disease.

**Increases in plasma chloride** are not nearly so important as are the reduced levels so far discussed. They are found occasionally in some types of nephritis, particularly in acute nephritis, and may also be seen in diseases of the urinary tract where there is obstruction to the flow of urine, for example, in enlarged prostate, though other factors in all these cases may operate to counteract this.

A tendency towards increased plasma chloride has been reported in Cushing's syndrome (hyperadrenalism) in which the increased secretion of the adrenal cortex gives an opposite set of changes to those found in Addison's disease.

## Dehydration

Owing to the close connection between chloride and water distribution in the body, chloride loss is accompanied by loss of water leading to dehydration. Estimation of plasma and urine chloride and of other serum electrolytes have been widely used in this state. Our knowledge of this subject has been greatly clarified during the last fifteen years since the work of McCance (1936, 1938) and has been surveyed by Marriott (1947, 1950) in a series of lectures which should be read by all concerned with this subject. See also Black (1951). Marriott stresses the importance of distinguishing between three types of dehydration.

(*a*) **Pure water depletion,** which occurs when insufficient water is taken without there being any significant loss of chloride. This may arise in illness when the patient is unable to swallow, as in dysphagia due to diphtheria, quinsy, carcinoma œsophagus, and in coma of any kind ; also whenever patients are in a state of such weakness that they cannot make their needs for water adequately known.

(*b*) **Pure salt depletion,** which occurs when salt and water are lost but only the water is replaced, and so may be found in any of the conditions described under chloride loss, in which adequate fluids are taken. Reduction of body water does occur, but only in an amount reflecting the diminished osmotic pressure consequent on the chloride loss.

(*c*) **Mixed salt and water depletion** may occur in those cases of (*b*) in which replacement of water is not adequately made. Here reduction of body water is in excess of that caused by the diminished osmotic pressure.

These lead to two different types of dehydration giving different symptoms and requiring different treatment. These have been termed intracellular and extracellular dehydration by Nadal and

his co-workers (1941, 1942) and primary and secondary dehydration by Marriott who now prefers the terms water and salt depletion.

The different symptoms arise from the differing effect on the extracellular fluid. In pure water depletion, the osmotic pressure of the extracellular fluid is increased, whereas in salt depletion it is reduced, so that the fluid is hypertonic and hypotonic respectively. In pure water depletion water is drawn from the cells, thus tending to maintain the volume of the extracellular fluid ; in salt depletion there is no such tendency and the kidneys excrete water in an attempt to keep up the osmotic pressure of the extracellular fluid. Hence in water depletion there is marked reduction in the volume of intracellular fluid with normal or but slightly reduced volume of the extracellular fluid ; in salt depletion intracellular fluid remains normal but there is marked reduction in the volume of extracellular fluid, a reduction which is greater in the interstitial fluid than in the plasma.

Among the results of water depletion are the following. Urine volume falls to a minimum, approximately 500 ml. if the kidneys are normal, and the specific gravity rises, exceeding 1·030 and even 1·040 on occasion. The plasma sodium chloride tends to rise, as does the blood urea, though slowly. There is usually an appreciable amount of chloride in the urine.

In salt depletion, on the other hand, provided water is being taken, urine volume remains normal and may even be increased in the earlier stages, though there is delayed excretion of ingested water. As the condition progresses there is a fall in plasma chloride which may reach 300 mg. per 100 ml. and even lower, whilst blood urea rises, sometimes very considerably. There is marked shrinkage in plasma volume, which tends to maintain normal plasma chloride levels even though considerable chloride loss has occurred. The consequence is that by the time plasma chloride is much reduced, chloride loss has been very great. As a result, absence of urine chloride is a more delicate index than plasma chloride and its estimation either by the approximate method of Fantus or by the accurate chemical method, gives valuable information both in diagnosis and in following treatment. According to Marriott if only 1 drop of silver nitrate is required in the Fantus test (p. 333), chloride should be regarded as "absent." This points to salt depletion only if diuresis is not occurring since it may be found in normal persons if very dilute urine is being passed. If the specific gravity is over 1·020, less than 3 drops, that is less than 3 grams per litre, suggests depletion whilst the patient is very unlikely to be suffering from salt depletion if the urine contains 5 grams per litre or over, Addison's disease being an exception.

In mixed water and salt depletion the symptoms are also mixed for while there is reduced volume of extracellular fluid due to loss of salt, the extra water loss, as in pure water depletion, tends to raise the osmotic pressure and may result in some water being withdrawn from the cells. Any of the conditions causing chloride loss may lead to such a mixed depletion if an adequate amount of water is not given.

The effects of vomiting and diarrhœa on the relative amounts of

sodium and chloride and bicarbonate ions has already been referred to. It is plain that the sodium level is a better index of the electrolyte loss than is the plasma chloride, since bicarbonate, which forms part of the total anion present in the plasma, may also be considerably altered, whereas sodium accounts for almost the whole of the cations. However, unless a flame photometer is available, chloride and bicarbonate determinations are more quickly, easily, and reliably made than is the estimation of sodium. But both together are really necessary for assessing the true loss of electrolyte. It is the sum of chloride and bicarbonate which should be used. If they are to be added, it is neeessary to express them in terms of milliequivalents per litre.

To convert mg. per 100 ml. into milliequivalents per litre it is only neeessary to multiply by 10 and divide by the equivalent weight, thus:

$$\text{milliequivalents per litre} = \frac{\text{mg. per 100 ml.} \times 10}{\text{equivalent weight}}.$$

Using this we can convert the amount of the various anions and cations present in the plasma into milliequivalents per litre and make it possible to add the two groups up and see what change has occurred either relatively or absolutely in them.

For the chief inorganic constituents the equivalent weights are :

| | |
|---|---|
| Sodium | 23 |
| Potassium | 39·1 |
| Calcium | 20 |
| Magnesium | 12 |
| Chloride | 35·5 (as Cl) ; 58·5 (as NaCl) |
| Phosphate (as phosphorus) | 31 ÷ 1·8 |
| Sulphate (as sulphur) | 32 ÷ 2 |
| Bicarbonate | 84 |

The factor 1·8 used for phosphate is derived from the proportion of dihydrogen and monohydrogen phosphate present at the *p*H of body fluids.

To convert alkali reserve expressed as volumes $CO_2$ per cent. (see p. 359) into milliequivalents per litre divide by 2·24.

Proteins are included with the anions in summing up the total equivalence to base. Van Slyke uses a factor of 2·43, so that milliequivalents per litre of protein

$$= \text{grams protein per 100 ml.} \div 2{\cdot}43.$$

The relative part played by the various constituents in maintaining the acid base balance is shown as follows (Gamble, 1947) :

| | | mEq./litre | | | mEq./litre |
|---|---|---|---|---|---|
| Base. | Na. | 142 | Acid. | $HCO_3$ | 27 |
| | K. | 5 | | Cl. | 103 |
| | Ca. | 5 | | $HPO_4$ | 2 |
| | Mg. | 3 | | $SO_4$ | 1 |
| | | | | Organic acids | 6 |
| | | | | Proteins | 16 |
| | | 155 | | | 155 |

Examples of the variations in the distribution of these electrolytes in several of the conditions discussed in this chapter are shown in diagrammatic form in Fig. 58, based on diagrams in Gamble's book *Chemical Anatomy, Physiology, and Pathology of Extracellular Fluid*,

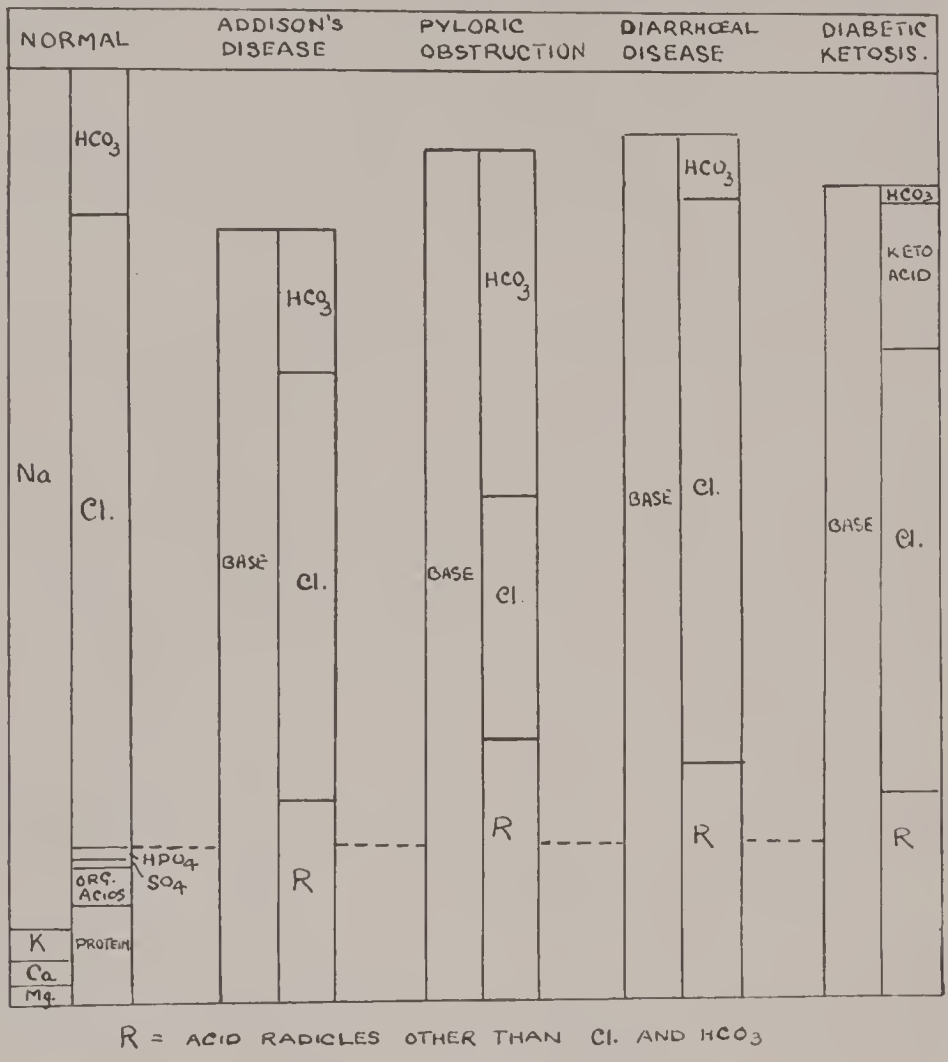


FIG. 58. Acid Base Balance.

Harvard University Press (Gamble, 1947), to which the reader is referred for a full discussion of this subject.

## POTASSIUM

**Determination of Serum Potassium.** Method of Jacobs and Hoffmann, (1931)

The potassium is precipitated directly from serum as a potassium sodium cobaltinitrite, $K_2NaCO(NO_2)_6$, $H_2O$. After suitable washing, the amount of cobalt in the precipitate is determined colorimetrically, using the green colour produced after the addition of choline chloride by the action of sodium ferrocyanide. A standard potassium solution may be treated similarly or a cobalt solution already standardized against a known potassium solution may be used directly.

**Reagents.** 1. Sodium cobaltinitrite reagent (Kramer and Tisdall, 1921). Prepare two solutions :

Solution A. Dissolve 25 grams of cobaltous nitrate in 50 ml. of water, and add to the solution 12·5 ml. of glacial acetic acid.

Solution B. Dissolve 120 grams of sodium nitrite (this should be potassium free) in 180 ml. of water. About 220 ml. of solution is obtained.

Add 210 ml. of solution B to all solution A. Evolution of nitric oxide occurs immediately. Draw air through the reagent until all the gas has been drawn off. A few hours may be required. Keep in a refrigerator and filter before using. The reagent will keep for about a month.

2. Ethyl alcohol, 70 per cent. (v/v).

3. Choline hydrochloride, 1 per cent. solution.

4. Sodium ferrocyanide, 2 per cent. solution.

5. Standard cobalt solution. Various cobalt salts have been used. Dissolve 0·372 gram of cobaltous nitrate, $Co(NO_3)_2$, $6H_2O$, or 0·359 gram of cobaltous sulphate, $CoSO_4$, $7H_2O$, or 0·506 gram of cobalt ammonium sulphate in water and make up to a litre. The latter salt recommended by King *et al.*. (1942) is the most convenient owing to the hygroscopic nature of the others. In any case the cobalt solution should be standardized against the standard potassium solution (20 mg. per 100 ml.) put through as described for the test. The above cobalt solutions are equivalent to 0·1 mg. potassium per ml. Once standardized the cobalt solutions keep well.

6. Stock potassium standard (10 mg. per ml.). Dissolve 2·228 grams of pure dry potassium sulphate in water and make up to 100 ml.

7. Standard potassium solution for use (20 mg. per 100 ml. or 0·2 mg. per ml.). Dilute 2 ml. of stock standard to 100 ml. with water.

**Technique.** Pipette 1 ml. of serum into a tapered centrifuge tube graduated at 6 ml. Add 2 ml. of sodium cobaltinitrite reagent slowly, shaking continuously. Stand for forty-five minutes, add 2 ml. of water, mix the contents well and centrifuge for fifteen minutes at a moderate speed. Remove the supernatant fluid by inverting the tube and allowing to drain on a filter paper for a short time. Run 1 ml. of water down the sides of the tube, centrifuge for five minutes, and again invert and drain. Then run in 2 ml. of 70 per cent. alcohol and mix with the precipitate by means of a stirring rod, centrifuge for five minutes, invert and drain. Repeat the washing with alcohol.

After the final draining, add 2 ml. of water and place in a boiling water bath for ten minutes, shaking frequently to dissolve the precipitate. A small amount of insoluble material, probably protein, may be left, but this does not interfere. Allow to cool and add first 1 ml. of 1 per cent. choline hydrochloride and then 1 ml. of 2 per cent. sodium ferrocyanide. Make up to the 6 ml. mark. At the same time treat 2 ml. of the cobalt standard in the same way, again making up to 6 ml. Compare the two solutions in the colorimeter. The colour is stable for hours. With the photoelectric colorimeter use an orange filter, or transmission at 620 millimicrons.

**Calculation.** Two ml. of cobalt solution are equivalent to 0·2 mg. of potassium and 1 ml. of serum was taken. For the photoelectric colorimeter :

Mg. potassium per 100 ml. serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}2 \times 100$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 20.$$

A standard curve can be made by treating varying amounts of a standardized cobalt solution with choline hydrochloride and sodium ferrocyanide, as follows :

| Mg. K per 100 ml. serum . . . | 0 | 4 | 8 | 12 | 16 | 20 | 25 |
|---|---|---|---|---|---|---|---|
| Ml. of cobalt standard (1 ml.=0·2 mg. K) | 0 | 0·2 | 0·4 | 0·6 | 0·8 | 1·0 | 1·25 |
| Ml. water . . . . . | 2·0 | 1·8 | 1·6 | 1·4 | 1·2 | 1·0 | 0·75 |

It should be noted that the cobalt solution is double the strength of that given above.

**Notes.** 1. All methods of estimating potassium are open to doubts as to the constancy of the composition of the precipitate formed. Carried out as described, this method appears to give a precipitate with a cobalt content sufficiently stable for the requirements of the test.

2. Barry and Rowland (1953) have recently studied the precipitation of potassium by cobaltinitrite from serum. They use the following technique which is claimed to give more complete precipitation. Add 3 ml. of the cobaltinitrite reagent to 1 ml. of serum and mix by rotating the tubes between the palms of the hands. Stand two hours at room temperature (15° C. or above), centrifuge ten minutes at 2,500 r.p.m. and decant the supernatant fluid. Wipe the mouth of the tube, add 5 ml. of 35 per cent. ethyl alcohol, washing down the sides of the tube. Mix with the precipitate using a fine glass rod. Centrifuge again for five minutes and decant. Repeat the washing twice. Add 3 ml. of water to the washed precipitate, immerse in boiling water, and mix until dissolved. Colour development is then carried out as described above.

3. In view of the small amount of potassium present in the serum and the large amount present in the cells it is obviously most important to avoid any hæmolysis and any diffusion of potassium out of the cells into the serum. The plasma potassium increases gradually when whole blood is allowed to stand. Accordingly non-hæmolyzed serum separated soon after taking the blood should be used.

4. Ammonium salts interfere since ammonium cobaltinitrite is also very insoluble. So any contamination with ammonium salts should be avoided. Also the estimation should be done on fresh serum and not on serum which may have stood for some time and become contaminated with bacteria.

**Other methods.** Looney and Dyer (1942) have used precipitation as potassium silver cobaltinitrite from a protein-free, chloride-free filtrate. Nitrite is liberated from the precipitate and estimated by means of the colour with sulphanilamide and N-(1-naphthyl) ethylene diamine dihydrochloride used in the Bratton and Marshall method for estimating sulphonamides.

Van Slyke and Rieben (1944) have described titrimetric and gravimetric methods using precipitation of potassium as the phospho-12-tungstate.

Potassium can be rapidly estimated by means of the flame photometer as indicated on p. 338.

INTERPRETATION

The normal serum potassium ranges from 15 to 22 mg. per 100 ml. (3·9 to 5·6 milliequivalents per litre). Much the greater part of the blood potassium is in the cells so that the potassium content of whole blood averages about 200 mg. per 100 ml. Whole blood potassium consequently varies considerably with the red cell count. Only serum potassium estimations have been used.

An *increase in serum potassium* is often found in severe Addison's disease (adrenal cortical insufficiency) particularly in the crises of that condition. Values up to and over 30 mg. per 100 ml. are usually found, but figures up to 40 to 50 have been reported. There is diminished excretion of potassium in the urine and a restricted intake of potassium benefits the condition. Increases have also been reported in uræmic coma and in acute intestinal obstruction.

A *decrease* in serum potassium is found during the attacks of paralysis occurring in patients with familial periodic paralysis. The potassium is more usually found in the region 10 to 12 mg. per 100 ml. but ranges from 8 to 14 mg. per 100 ml. The condition is relieved by giving potassium chloride. A low serum potassium may be produced by severe diarrhœa and vomiting, and has been reported in Cushing's syndrome.

Injection of adrenaline is followed by a temporary rise, injection of insulin in considerable dosage by a temporary fall in serum potassium. The very low values which may be produced in this way during the treatment of diabetic coma have been referred to previously (p. 81).

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## *CHAPTER XIX*

# ACID-BASE BALANCE

The body has a number of methods available by which it can keep the *p*H of the blood within normal limits. These make use of several substances present in blood, which can neutralize the effect of any influx of acidic or alkaline products, and of the excretion, particularly by the lungs and kidneys, of some of the substances produced by these buffering reactions. Important in this connection are the following.

1. The blood **bicarbonate.** There are present in blood both the weak base, bicarbonate, and the weak acid, carbonic acid Stronger acids react with bicarbonate to form the weak carbonic acid, whilst alkalies react with carbonic acid to form bicarbonate, so that there is little change in *p*H in either case Since carbon dioxide is continuously being produced in the body and being removed by the lungs, this buffering system is a most important one. The carbon dioxide formed by neutralization of bicarbonate can be rapidly removed by the lungs, and carbon dioxide is readily available to neutralize alkalies to form bicarbonate which can be excreted by the kidneys.

2. **Phosphates.** The phosphate present in body fluids is a mixture of the monohydrogen and dihydrogen salts, the former being weakly alkaline and the latter weakly acidic. Accordingly relatively little change in *p*H results from the conversion of one to the other, yet an equivalent amount of a strong acid or base is required to bring this about, for example,

$$Na_2HPO_4 + HCl = NaCl + NaH_2PO_4$$

This system is more important in the tissues than in the blood, in which it plays only a minor part.

3. The **plasma proteins.** At the *p*H of blood plasma these play the part of weak acids and can combine with some base. However, this buffering power is small compared with that of the other systems.

4. The **red cells** also play an important part in *p*H regulation. Several processes contribute to this. Hæmoglobin, as a weak acid, is mainly combined with potassium in the cells. It is, however, a stronger acid as oxyhæmoglobin than as reduced hæmoglobin, and so takes up or releases base as oxygenation varies. Carbon dioxide can pass into the cell where carbonic anhydrase catalyses formation of carbonic acid. Chloride is also involved since the ratio of $HCO_3$ ions in cells and plasma equals that of Cl ions in cells and plasma. The chloride shift which results from transfer of carbonic acid and formation of carbonic acid in the cells alters the relative amount of chloride and bicarbonate and so influences the *p*H.

5. The **kidneys** are mainly important in conserving base. Two processes are particularly important. By excreting a more acid urine, down to a *p*H of 4·8, less base is lost, since in the case of

phosphate the salt excreted is the monosodium instead of the disodium, and in the case of ketosis, for example, a greater proportion of the $\beta$-hydroxybutyric acid is excreted as the acid than as the salt. Also, by forming ammonia and excreting ammonium salts instead of sodium salts fixed base is conserved.

6. The part played by the **lungs** in removing carbon dioxide has been referred to above.

For a more complete account of the maintenance of the normal acid-base balance larger works of physiology should be consulted. Here we shall be concerned mainly with variations in the bicarbonate carbonic acid system. Tests based on alterations in this are most frequently used in studying disturbances in acid-base balance. The term alkali reserve, that is the base available for neutralizing acids produced in the body, is commonly used as being equivalent to the plasma bicarbonate.

## Determination of the Plasma Bicarbonate

### I. Gasometric Method of Van Slyke and Cullen (1917)

Blood is taken under liquid paraffin and the plasma immediately separated by centrifuging. The plasma is shaken with expired air so that it takes up as much carbon dioxide as it can hold. A known volume of this plasma is acidified to liberate the carbon dioxide from the bicarbonate present. A partial vacuum is produced, and the volume of carbon dioxide liberated, is measured. This consists of the carbon dioxide derived from the bicarbonate and that—usually a much smaller amount—in physical solution. In the calculation this latter is subtracted so that the volume reported gives a measure of the bicarbonate in the plasma examined.

**Apparatus.** The apparatus used is that of Van Slyke and is shown in Fig. 59. This consists of a 50 ml. pipette with a two-way tap at each end. The upper part is a tube of just over 1 ml. capacity about 5 to 6 inches in length calibrated from the top downwards in 0·02 ml. divisions. This widens out a little at the lower end and has the 2·5 ml. mark just before the bulb of the pipette is reached. On the lower side of the bulb is the 50 ml. mark on the very short neck between the bulb and the lower stop tap B. The upper two-way tap A connects the pipette with a cup C used for introducing liquids into the pipette, and with the side tube D used for removing liquids from the apparatus. The lower tap B connects the pipette with two tubes, E and F, into the wider tube F of which the liquid can be withdrawn after the carbon dioxide has been liberated, the mercury then being readmitted through the narrower tube E into the pipette. Thick rubber tubing is used to join the lower end of this apparatus to a reservoir of mercury. The whole apparatus should be firmly held with a strong clamp gripping the bulb of the pipette and an iron rod covered with rubber should give support by being placed under the stop tap B. It is important to see that the stop taps are well greased and airtight. They should be held in position with strong rubber

bands, or be of the type with small springs attached so as to prevent the taps from being forced out by the pressure of the mercury. There is also a good deal to be said for fastening the base of the tripod to the bench. Three retort rings should be fixed in positions such that the mercury reservoir can be placed (1) just above the level of tap A, (2) just below the level of the 2·5 ml. mark on the pipette, and (3) about 80 cm. below the level of the lower tap B which should bring the mercury level in the apparatus to about the middle of E.

The apparatus should always be carefully tested immediately before use to prove that it is airtight and free from gases. To do this, fill the pipette completely with mercury and force a little into each of C and D. Turn tap A at right angles and lower the mercury reservoir to well below the level of tap B with this tap open. When the bulb is raised again the mercury should completely refill the pipette and should strike the stop tap A with a smart click.

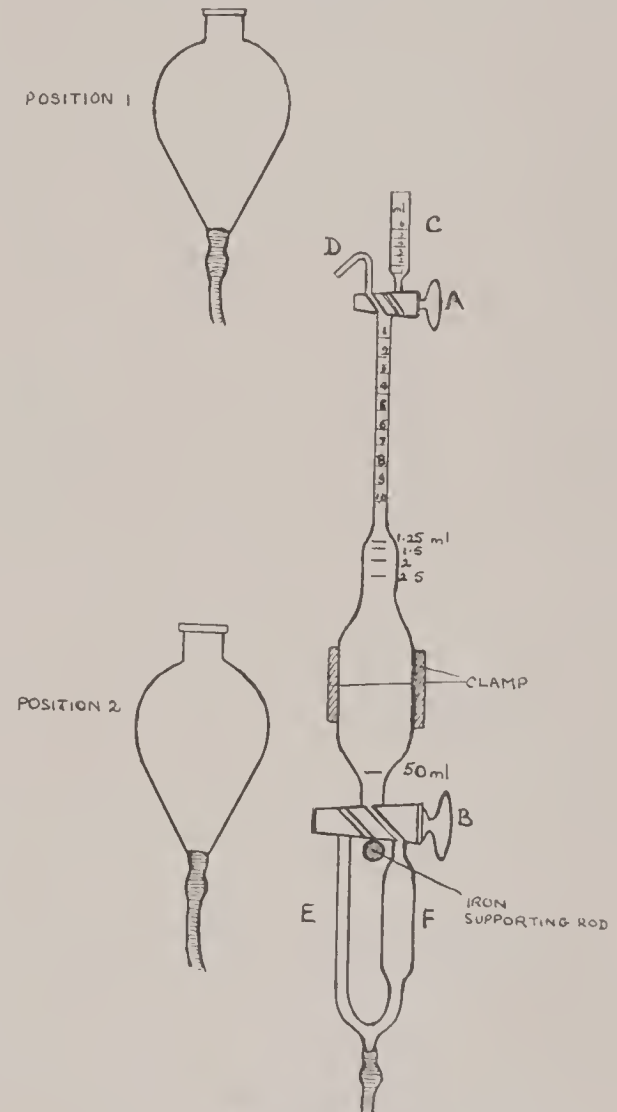


Fig. 59. Van Slyke's Apparatus.

**Reagents.** 1. Lactic acid. Dilute the concentrated acid (specific gravity 1·25) 1 to 10 with water. Alternatively, tartaric acid as a 20 per cent. aqueous solution, or 5 per cent. sulphuric acid (v/v) may be used. The organic acids are to be preferred, particularly if whole blood is used.

2. Caprylic alcohol.

**Technique.** The blood should be withdrawn without stasis into a syringe containing a little liquid paraffin and then placed into a centrifuge tube containing some liquid paraffin together with some finely powdered potassium oxalate (see p. 6). Rotate the tube to mix the blood with the oxalate and then centrifuge. Remove the plasma. This may be kept if desired, but if so, bacterial action must be prevented.

The plasma is next saturated with carbon dioxide by shaking with alveolar air. Place about 3 ml. of the plasma into a clean dry separating funnel of about 250 ml. capacity which has been connected as shown in Fig. 60 to a bottle full of glass beads which holds back the moisture in the expired air. Apply the mouth to the mouth-

piece, and, breathing normally, expire quickly and as completely as possible. Insert the stopper into the funnel just before the end of the expiration. Close the tap, disconnect the funnel and rotate for a few minutes so that the plasma is spread out in as thin a layer as possible on the walls. Then stand upright and allow the plasma to drain into the bottom of the funnel.

In determining the carbon dioxide which can be liberated, fill the apparatus with mercury, allowing a little to go through into both C and D. Shut off tap A. Wash the cup C with water or carbonate-free dilute ammonia in order to remove any traces of acid. Run 1 to 2 ml. of distilled water into the cup, and add a small drop of caprylic alcohol to reduce the frothing which may occur in the pipette later. With the tip of the pipette below the level of the water in C, run in 1 ml. of the plasma from the separating funnel. With care it is possible to run this in so that it forms a sharply

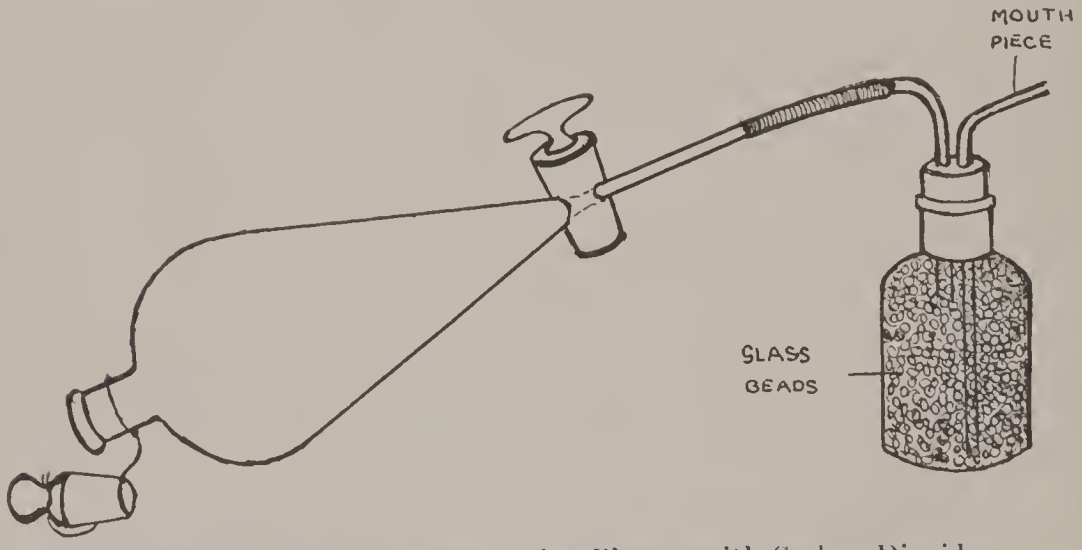


Fig. 60. Apparatus for saturating Plasma with Carbon Dioxide.

defined layer below the water. With the reservoir in position 2 or held somewhere between positions 1 and 2, allow the plasma to run slowly into the pipette by cautiously opening the tap A. Allow the whole of the plasma and about 0·5 ml. of water to run in before closing the tap again. Then, in the same way, run 0·5 ml. of the acid into the cup underneath the water. Again cautiously opening the tap, allow the acid to run slowly into the pipette followed by enough water to bring the total volume of liquid in the pipette to the 2·5 ml. mark. Close the tap by turning at right angles, and seal off by placing a small amount of mercury in the bottom of the cup C. The amount of acid required is only approximately 0·5 ml., but the total volume of liquid in the pipette should be exactly 2·5 ml.

Lower the mercury reservoir to the position about 80 cm. below the lower tap B. Allow the mercury level to run down to the 50 ml. mark and close tap B. Remove the pipette from its clamp and invert about 15 to 20 times, or use a shaking apparatus, and so bring the carbon dioxide into equilibrium between the 2·5 ml. of solution

and the 47·5 ml. of free space. Replace the pipette in the clamp and by carefully turning tap B, run the mercury and liquid completely into chamber F without letting in any of the gas. Then turn the tap B so that on raising the reservoir the mercury returns into the pipette through the tube E. Read the volume of carbon dioxide liberated in the calibrated part of the pipette by levelling off the mercury in the reservoir and pipette. If a small amount of the plasma-acid mixture remains above the mercury level in the pipette, hold the mercury level in the reservoir at a height above the mercury level in the pipette corresponding to one-thirteenth of the height of the aqueous layer.

Wash out the apparatus with distilled water after each determination. Run the plasma-acid mixture through the side tube D and then admit distilled water from the cup C, mix well with the mercury by lowering the reservoir down to the lowest ring. After a time, however, the apparatus becomes contaminated with bits of grease and protein, and more thorough cleaning is necessary. Strong alkali can be introduced as washing agent. It is desirable occasionally to dismantle and clean thoroughly.

If the volume of gas evolved is more than 1 ml. in volume, or if the volume of plasma available is less than 1 ml., carry out the test on 0·5 ml. of plasma, using half quantities throughout, that is, using a total volume of 1·25 ml. instead of 2·5 ml. The observed volume of carbon dioxide is then multiplied by 2 and the alkali reserve calculated as described below.

**Calculation.** An approximate result can be obtained by multiplying this volume of carbon dioxide by 100, and subtracting 12. This gives the alkali reserve in ml. of carbon dioxide per 100 ml. of plasma. The possible error introduced by using such an approximation will be of the order of $\pm$ 3 ml. carbon dioxide per 100 ml. plasma, for the usual range of temperature and pressure. Such an error is not serious from a clinical standpoint, so that this simplified calculation may be used for ordinary purposes.

Otherwise the temperature and barometric pressure must be measured. The volume of carbon dioxide liberated is multiplied by

TABLE XVIII

*Factors for Multiplying by B/760*

| B | B/760 | B | B/760 | B | B/760 |
|---|---|---|---|---|---|
| 730 | 0·961 | 746 | 0·982 | 762 | 1·003 |
| 732 | 0·963 | 748 | 0·984 | 764 | 1·005 |
| 734 | 0·966 | 750 | 0·987 | 766 | 1·008 |
| 736 | 0·968 | 752 | 0·989 | 768 | 1·011 |
| 738 | 0·971 | 754 | 0·992 | 770 | 1·013 |
| 740 | 0·974 | 756 | 0·995 | 772 | 1·016 |
| 742 | 0·976 | 758 | 0·997 | 774 | 1·019 |
| 744 | 0·979 | 760 | 1·000 | 776 | 1·021 |

B/760 where B is the barometric pressure, in mm. of mercury. This corrected volume is then used for reading the chemically bound carbon dioxide directly from Table XIX. A table is also given from which B/760 can be read.

## Table XIX

*For Calculation of Carbon Dioxide Combining Power of Plasma* (Van Slyke and Cullen, 1917)

| Observed vol. gas $\times \frac{B}{760}$ | Ml. $CO_2$ reduced to 0° C., 760 mm. bound as bicarbonate by 100 ml. plasma | | | | Observed vol. gas $\times \frac{B}{760}$ | Ml. $CO_2$ reduced to 0° C., 760 mm. bound as bicarbonate by 100 ml. plasma | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 15° | 20° | 25° | 30° | | 15° | 20° | 25° | 30° |
| 0·20 | 9·1 | 9·9 | 10·7 | 11·8 | 0·60 | 47·7 | 48·1 | 48·5 | 48·6 |
| 1 | 10·1 | 10·9 | 11·7 | 12·6 | 1 | 48·7 | 49·0 | 49·4 | 49·5 |
| 2 | 11·0 | 11·8 | 12·6 | 13·5 | 2 | 49·7 | 50·0 | 50·4 | 50·4 |
| 3 | 12·0 | 12·8 | 13·6 | 14·3 | 3 | 50·7 | 51·0 | 51·3 | 51·4 |
| 4 | 13·0 | 13·7 | 14·5 | 15·2 | 4 | 51·6 | 51·9 | 52·2 | 52·3 |
| 5 | 13·9 | 14·7 | 15·5 | 16·1 | 5 | 52·6 | 52·8 | 53·2 | 53·2 |
| 6 | 14·9 | 15·7 | 16·4 | 17·0 | 6 | 53·6 | 53·8 | 54·1 | 54·1 |
| 7 | 15·9 | 16·6 | 17·4 | 18·0 | 7 | 54·5 | 54·8 | 55·1 | 55·1 |
| 8 | 16·8 | 17·6 | 18·3 | 18·9 | 8 | 55·5 | 55·7 | 56·0 | 56·0 |
| 9 | 17·8 | 18·5 | 19·2 | 19·8 | 9 | 56·5 | 56·7 | 57·0 | 56·9 |
| 0·30 | 18·8 | 19·5 | 20·2 | 20·8 | 0·70 | 57·4 | 57·6 | 57·9 | 57·9 |
| 1 | 19·7 | 20·4 | 21·1 | 21·7 | 1 | 58·4 | 58·6 | 58·9 | 58·8 |
| 2 | 20·7 | 21·4 | 22·1 | 22·6 | 2 | 59·4 | 59·5 | 59·8 | 59·7 |
| 3 | 21·7 | 22·3 | 23·0 | 23·5 | 3 | 60·3 | 60·5 | 60·7 | 60·6 |
| 4 | 22·6 | 23·3 | 24·0 | 24·5 | 4 | 61·3 | 61·4 | 61·7 | 61·6 |
| 5 | 23·6 | 24·2 | 24·9 | 25·4 | 5 | 62·3 | 62·4 | 62·6 | 62·5 |
| 6 | 24·6 | 25·2 | 25·8 | 26·3 | 6 | 63·2 | 63·3 | 63·6 | 63·4 |
| 7 | 25·5 | 26·2 | 26·8 | 27·3 | 7 | 64·2 | 64·3 | 64·5 | 64·3 |
| 8 | 26·5 | 27·1 | 27·7 | 28·2 | 8 | 65·2 | 65·3 | 65·5 | 65·3 |
| 9 | 27·5 | 28·1 | 28·7 | 29·1 | 9 | 66·1 | 66·2 | 66·4 | 66·2 |
| 0·40 | 28·4 | 29·0 | 29·6 | 30·0 | 0·80 | 67·1 | 67·2 | 67·3 | 67·1 |
| 1 | 29·4 | 30·0 | 30·5 | 31·0 | 1 | 68·1 | 68·1 | 68·3 | 68·0 |
| 2 | 30·3 | 30·9 | 31·5 | 31·9 | 2 | 69·0 | 69·1 | 69·2 | 69·0 |
| 3 | 31·3 | 31·9 | 32·4 | 32·8 | 3 | 70·0 | 70·0 | 70·2 | 69·9 |
| 4 | 32·3 | 32·8 | 33·4 | 33·8 | 4 | 71·0 | 71·0 | 71·1 | 70·8 |
| 5 | 33·2 | 33·8 | 34·3 | 34·7 | 5 | 71·9 | 72·0 | 72·1 | 71·8 |
| 6 | 34·2 | 34·7 | 35·3 | 35·6 | 6 | 72·9 | 72·9 | 73·0 | 72·7 |
| 7 | 35·2 | 35·7 | 36·2 | 36·5 | 7 | 73·9 | 73·9 | 74·0 | 73·6 |
| 8 | 36·1 | 36·6 | 37·2 | 37·4 | 8 | 74·8 | 74·8 | 74·9 | 74·5 |
| 9 | 37·1 | 37·6 | 38·1 | 38·4 | 9 | 75·8 | 75·8 | 75·8 | 75·4 |
| 0·50 | 38·1 | 38·5 | 39·0 | 39·3 | 0·90 | 76·8 | 76·7 | 76·8 | 76·4 |
| 1 | 39·1 | 39·5 | 40·0 | 40·3 | 1 | 77·8 | 77·7 | 77·7 | 77·3 |
| 2 | 40·0 | 40·4 | 40·9 | 41·2 | 2 | 78·7 | 78·6 | 78·7 | 78·2 |
| 3 | 41·0 | 41·4 | 41·9 | 42·1 | 3 | 79·7 | 79·6 | 79·6 | 79·2 |
| 4 | 42·0 | 42·4 | 42·8 | 43·0 | 4 | 80·7 | 80·5 | 80·6 | 80·1 |
| 5 | 42·9 | 43·3 | 43·8 | 43·9 | 5 | 81·6 | 81·5 | 81·5 | 81·0 |
| 6 | 43·9 | 44·3 | 44·7 | 44·9 | 6 | 82·6 | 82·5 | 82·4 | 82·0 |
| 7 | 44·9 | 45·3 | 45·7 | 45·8 | 7 | 83·6 | 83·4 | 83·4 | 82·9 |
| 8 | 45·8 | 46·2 | 46·6 | 46·7 | 8 | 84·5 | 84·4 | 84·3 | 83·8 |
| 9 | 46·8 | 47·1 | 47·5 | 47·6 | 9 | 85·5 | 85·3 | 85·2 | 84·8 |
| 0·60 | 47·7 | 48·1 | 48·5 | 48·6 | 1·00 | 86·5 | 86·2 | 86·2 | 85·7 |

INTERPRETATION

The above method gives the ml. of carbon dioxide which are combined as bicarbonate in 100 ml. plasma at 0° C. and 760 mm. mercury. The capacity of the plasma to combine with carbon dioxide depends on the amount of alkali available and so is a measure of the alkali reserve. Strictly speaking the term alkali reserve applies to all the base which is available for removing relatively strong acids formed or introduced into the body, thus keeping the hydrogen ion concentration of the plasma within normal limits. It includes base combined with other substances such as phosphates, plasma proteins, hæmoglobin. But this use of the term alkali reserve to refer to the plasma bicarbonate is sufficiently accurate for clinical purposes.

The normal range for the alkali reserve by this method is from 53 to 75 ml. of carbon dioxide per 100 ml. plasma, sometimes referred to as volumes per cent. Expressed as mg. bicarbonate per 100 ml. plasma this is 200 to 285 ; or 24 to 35 milliequivalents per litre. Both increase and decrease occur in pathological conditions. These may either be due to a primary change in plasma bicarbonate, or be secondary to a change in the carbonic acid content of the blood. According to whether these changes produce or tend to produce a decrease or increase in the plasma *p*H they lead to an acidosis or an alkalosis respectively. The *p*H is influenced by both the concentration of carbonic acid and of bicarbonate, thus

$$\text{H ion concentration} \propto \frac{\text{Carbonic acid concentration } [H_2CO_3]}{\text{Bicarbonate concentration } [BHCO_3]}$$

We see that there are four possibilities, as follows :

A. Acidosis may result from

1. Primary loss of bicarbonate.
2. Primary increase in the carbonic acid content of the plasma.

Both these changes tend to produce an increase in H ion concentration in the plasma, and to decrease the *p*H.

B. Alkalosis may result from

1. Primary increase in plasma bicarbonate.
2. Primary decrease in the carbonic acid content of the plasma.

These changes tend to produce a decrease in H ion concentration of the plasma, and to increase the *p*H.

It should be noted that whenever one of these primary changes occurs there is an attempted compensatory change in the other factor. Thus primary loss of bicarbonate is accompanied by a secondary decrease in plasma carbonic acid brought about by an increased rate of breathing which washes out more carbon dioxide from the alveolar air, whilst primary increase in bicarbonate leads to a reduced rate of breathing with increase in plasma carbonic acid. On the other hand, primary increase in carbonic acid content leads to compensatory increase in plasma bicarbonate, primary decrease in carbonic acid to decreased plasma bicarbonate. As a result of these compensatory changes, when there are slight to moderate variations in bicarbonate,

plasma *p*H is kept within normal limits. With marked alterations, however, compensation is inadequate and changes in *p*H result.

It is thus seen that abnormal plasma bicarbonate may occur in all the four groups of conditions given above.

**Diminished Alkali Reserve.** 1. *Primary Loss of Bicarbonate.* The two conditions in which the lowest values for plasma alkali reserve are found are diabetic coma and uræmic coma, in both of which it may fall to below 10 volumes per cent. Coma is usually present when a figure below 15 is reached. From normal to 30 volumes per cent. may be regarded as showing a mild to moderate degree of acidosis, usually adequately compensated ; below 30 the acidosis is an increasingly severe one.

Starvation may lead to a ketosis similar to that of diabetes and to a reduced alkali reserve. Dehydration resulting from loss of a considerable amount of fluid from the intestine, as, for example, in diarrhœa, may lead to a moderate reduction in alkali reserve to between 40 and 50 volumes per cent., since there is bicarbonate in the secretions into the small intestine. There is also some reduction in toxæmia of pregnancy and a tendency for there to be a slight fall in normal pregnancy.

An acidosis arising from defective reabsorption of bicarbonate in the renal tubules has been recognized. The plasma bicarbonate is reduced, the alkali reserve being between normal and about 20 volumes per cent. (about 10 milliequivalents per litre). This is accompanied by an increase in plasma chlorides. The condition is termed hyperchloræmic renal acidosis (see Latner and Burnard, 1950 ; Milne *et al.*, 1952).

2. *Primary Decrease in Plasma Carbonic Acid. Secondary Decrease in Plasma Bicarbonate.* This arises whenever there is hyperventilation due to an increased rate of breathing so that abnormally large quantities of carbon dioxide are washed out of the blood. This may occur physiologically at high altitudes and clinically, for example, in cases of encephalitis and in hysteria. The compensatory fall in bicarbonate produces an alkali reserve between 40 and 50 volumes per cent.

**Increased Alkali Reserve.** 1. *Primary Increase in Bicarbonate.* The commonest cause of this is the excessive loss of hydrochloric acid in the vomiting which occurs in cases of pyloric stenosis or spasm associated most often with duodenal ulcer. The base set free is combined as bicarbonate thus producing a high plasma bicarbonate. When the increase is small, to 80 to 90 volumes per cent., a compensatory change in plasma carbonic acid usually keeps plasma *p*H within normal limits, but is not able to do so in severer cases in which the alkali reserve may reach 100 to 120 volumes per cent.

Ingestion of large amounts of bicarbonate may also raise the alkali reserve considerably, particularly if there is some renal impairment.

2. *Primary Increase in Plasma Carbonic Acid. Secondary Increase in Plasma Bicarbonate.* This may be due to a less efficient removal of carbon dioxide from the lungs. The tendency towards a mild

acidosis is compensated by a small to moderate increase in plasma bicarbonate, with alkali reserve up to 80 to 90 volumes per cent. This may be seen in pneumonia, emphysema, in some heart conditions in which there is lung congestion, and in asphyxia.

Tetany, which we have already seen occurs when there is reduced serum calcium (p. 297), may also develop in alkalosis, and so may be found in conditions in which there is primary decrease in plasma carbonic acid or primary increase in plasma bicarbonate.

### II. Van Slyke Titration Method (Van Slyke, 1922)

Plasma bicarbonate has also been estimated by direct titration of the plasma. This is not so easy to carry out as the above and has not been as much used. The results of the two methods naturally parallel each other. In Van Slyke's form of the titration method an excess of acid is added to plasma from blood collected as before under paraffin. The mixture is then titrated back to the *p*H of the original plasma using plasma + saline + indicator as control.

**Reagents.** 1. Sodium chloride, 0·9 per cent. solution.

2. N/100 hydrochloric acid. Prepare by diluting stock N/10 acid with 0·9 per cent. sodium chloride solution.

3. N/100 carbonate-free sodium hydroxide solution. Prepare from stock carbonate-free N/10 solution by diluting with 0·9 per cent. sodium chloride. Prepare freshly before use.

4. Stock 0·1 per cent. phenol red solution. Take 0·1 gram of the indicator and 4·1 ml. of N/20 sodium hydroxide and make to 100 ml. with water. Dilute 3 ml. to 10 ml. with water to get the 0·03 per cent. solution.

5. Phenol red solution, *p*H 7·4. Add 35 drops of 0·03 per cent. phenol red solution to 100 ml. of 0·9 per cent. sodium chloride. Adjust to *p*H 7·4 by means of N/100 sodium hydroxide. Test the *p*H by comparing with 15 ml. of a phosphate standard of *p*H 7·4 to which 5 drops of 0·03 per cent. phenol red have been added. Cover the solution, when prepared, with a layer of liquid paraffin. Prepare freshly before use.

**Technique.** Take the blood under paraffin as described previously into an oxalate tube containing paraffin, and centrifuge immediately. Remove the plasma and keep under oil. Pipette 1 ml. of this and add to 20 ml. of the saline-phenol red solution under paraffin in a test tube. Pipette another ml. of plasma into a small round-bottomed flask and add 5 ml. of N/100 hydrochloric acid. Whirl the flask about for at least one to two minutes to allow the carbon dioxide to escape. Then pour the solution into a test tube similar to the one already used, washing in with 10 ml. of 0·9 per cent. sodium chloride. Add 7 drops of the phenol red solution and titrate with N/100 sodium hydroxide until the colour matches that of the standard tube. The final colour comparison should not be made until at least thirty seconds after the addition of the last drop of alkali, since as the end point is approached, each drop of alkali appears to send the colour past the end point but shifts back after a few seconds. Another drop has then to be added. This is continued until the test and

standard match at least thirty seconds after the last addition of alkali.

Carry out a blank titration on 5 ml. of the N/100 acid to which 10 ml. of 0·9 per cent. sodium chloride and 7 drops of phenol red have been added.

All the tubes used should be scrupulously cleaned.

**Calculation.** If T ml. N/100 sodium hydroxide are required for the test and B ml. (approximately 5) for the blank, then there are (B — T) ml. of N/100 or M/100 sodium bicarbonate per ml. of plasma, that is (B — T) ml. of N or M bicarbonate per 100 ml. plasma. To convert into ml. of carbon dioxide per 100 ml. plasma we need only to multiply this by 22·4, since 1 molecular weight in grams of bicarbonate gives 1 molecular weight in grams of carbon dioxide and this occupies 22·4 litres. One litre of M/1 bicarbonate contains 1 molecular weight of the salt and so produces 22·4 litres of carbon dioxide. Hence 1 ml. of M/1 $NaHCO_3$ gives 22·4 ml. So ml. carbon dioxide per 100 ml. plasma = (B — T) in ml. × 22·4.

### III. Approximate Capillary Method of Wootten and King (1949)

This is a rapid approximate method which may be useful in certain circumstances.

**Reagents.** 1. Chlorophenol red solution. 0·04 per cent. in water.

2. Standard acids. Prepare a series of standard acids by diluting N/10 acid.—N/10, N/12, N/15, N/17, N/20, N/25, N/30 . . . N/55 are suggested. Keep in waxed bottles,

3. Set of British Drug Houses capillator standards for chlorophenol red.

**Technique.** Prepare a small Pasteur type pipette and graduate at about 1 inch and 2 inches along the capillary from the end, so that the volume to mark 2 is twice that at mark 1. Measure the chlorophenol red indicator up to mark 2 and place on a small watch glass. Add one of the standard acids to mark 1 and then 1 volume of the plasma which has been shaken with alveolar air. Mix well and measure the *p*H by drawing up into the capillary tube and comparing with the capillator standards. Find the acid which gives a final *p*H of 5·5.

**Calculation.** Carbon dioxide combining power, in volumes per cent. = 1,165 × Normality of the acid required to reduce the *p*H to 5·5. The factor 1,165 was determined empirically by comparison with the gasometric methods.

Example. If N/20 acid is required, the alkali reserve is 1,165/20, that is 58 volumes per cent.

Standard deviation from the gasometric method is ± 4 volumes per cent. A modification of this method has been described by Lehmann (1951).

### Carbon Dioxide Content of Alveolar Air

Whilst determination of the alkali reserve is the test most frequently carried out in connection with acid-base equilibrium of the blood, others have been used and will be referred to now.

Determination of blood carbonic acid can be made but is rarely done. More often an indication of its amount is obtained by measuring the carbon dioxide content of the alveolar air which is in equilibrium with the carbon dioxide of the arterial blood. This is of most value in conditions where the acid-base disturbance is due to a primary alteration in the blood carbonic acid.

On breathing out it has been shown that an expiration of 400 ml. is sufficient to wash out completely the respiratory dead space so that further expired air can be regarded as genuine alveolar air. Accordingly the patient breathes out normally and then follows this with a forced expiration to give alveolar air for analysis.

In the method of Haldane and Priestley (1904–05) a sample of such air is collected and analysed in the Haldane gas analysis apparatus. For collecting the specimen of air, they used a long straight tube about a metre long and 2 cm. diameter which had a capillary side tube near one end through which a sample could be obtained. This tube was fitted with a piece of hose about 4 feet long. Boothby and Peabody (1914) fitted a three-way valve in between this tube and the patient. For a different apparatus, see Henderson and Morris (1917).

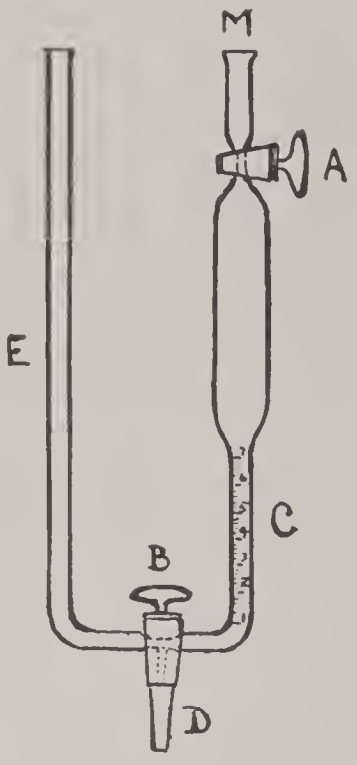

Fig. 61. Fridericia's Apparatus.

The *Fridericia apparatus* (Poulton, 1915) combines both collection and analysis. It is less accurate theoretically than the Haldane method but the error thus introduced is not appreciable clinically or when compared with successive estimations using the more accurate methods. This apparatus is shown in Fig. 61. The capacity of the tube and bulb between taps A and B is 100 ml., the tube C having about 10 ml. volume and being calibrated in 0·1 ml., or in 0·1 per cent. of the total volume if this is not exactly 100 ml.

Wash the apparatus out with 5 per cent. acetic acid before use. When using, hold the apparatus with tap A open and with tap B arranged so that E connects with C. At the beginning of an expiration place the mouth at the end of tube M and breathe out as completely as possible through the apparatus which is thus washed out and left full of alveolar air. Close tap A at the end of the expiration and place the apparatus in water at room temperature for five minutes. This cools the air in the apparatus to room temperature at which the remainder of the test will be carried out. Some contraction of air occurs drawing into C some of the air in E. At the end of the five minutes turn tap B to connect E with D. Then dip D into a 10 per cent. sodium hydroxide solution, some of which is carefully drawn up into E by means of a rubber teat on the end of E. Turn tap B to connect E with C and force some of the sodium

hydroxide into C. Then immediately turn the tap so as to connect D and E and force the alkali run out of E. By inverting several times, the carbon dioxide in the air in C is absorbed by the alkali. Replace the apparatus in the water which then rises through D into E. Turn the tap to connect C with D and leave in the water for five minutes. Raise the apparatus until the water in the calibrated tube is at the same level as the water in the bath. Turn the tap to join D and E and take out of the water and read the level in C which thus gives directly the per cent. of carbon dioxide in the alveolar air.

INTERPRETATION

The normal range by this method gives 4·7 to 5·1 volumes of carbon dioxide per cent. for men and from about 4·2 to 4·6 for women. Low values are found in alkalosis due to primary decrease in plasma carbonic acid, for example, in hyperventilation ; and in acidosis as a secondary change when the blood carbonic acid is lowered by a compensatory hyperventilation accompanying a primary reduction in plasma bicarbonate. Values then range from 4·8 to 3·2 in mild acidosis, from 3·2 to 2·3 in moderate to severe acidosis, and below 2·3 in severe acidosis. Increases are obtained in acidosis due to primary increase in blood carbonic acid arising from deficient removal of carbon dioxide from the lungs, as in asphyxia. Increase may also occur as a compensatory phenomenon when there is alkalosis arising from primary increase in plasma bicarbonate. Diminished pulmonary ventilation leads to reduced elimination of carbon dioxide from the lungs, retention of carbonic acid in the blood and increased concentration in the alveolar air.

## Determination of the Hydrogen Ion Concentration of the Plasma

This may be done by electrometric or colorimetric methods. Relatively simple colorimetric methods can be used if a *p*H meter is not available.

### Colorimetric Method of Cullen and Hawkins (Cullen, 1922 ; Hawkins 1923)

Plasma obtained from blood collected as described under alkali reserve is mixed with a phenol red-salt solution and matched against standard phosphate-phenol red mixtures of known *p*H.

**Reagents.** 1. Sodium chloride, 0·9 per cent. solution.

2. Phenol red-salt solution, *p*H 7·4. Prepare as in the titration method for plasma bicarbonate, p. 361.

3. Phosphate standards. Sörensen's *p*H standards are used. Prepare M/15 solutions of disodium hydrogen phosphate (11·87 grams of $Na_2HPO_4$, $2H_2O$ or 9·47 grams of the anhydrous salt in a litre of solution) and of potassium dihydrogen phosphate (9·08 grams of the anhydrous salt per litre of solution). Mix as shown on the next page for the *p*H range to be covered.

Prepare the standards by adding to 15 ml. of each of these, 5 drops (0·3 ml.) of a 0·03 per cent. phenol red solution, as prepared, p. 361.

| *p*H | M/15 $Na_2HPO_4$ ml. | M/15 $KH_2PO_4$ ml. | *p*H | M/15 $Na_2HPO_4$ ml. | M/15 $KH_2PO_4$ ml. |
|---|---|---|---|---|---|
| 7·00 | 61·1 | 38·9 | 7·45 | 82·5 | 17·5 |
| 7·05 | 63·9 | 36·1 | 7·50 | 84·1 | 15·9 |
| 7·10 | 66·6 | 33·4 | 7·55 | 85·7 | 14·3 |
| 7·15 | 69·2 | 30·8 | 7·60 | 87·0 | 13·0 |
| 7·20 | 72·0 | 28·0 | 7·65 | 88·2 | 11·8 |
| 7·25 | 74·4 | 25·6 | 7·70 | 89·4 | 10·6 |
| 7·30 | 76·8 | 23·2 | 7·75 | 90·5 | 9·5 |
| 7·35 | 7·89 | 21·1 | 7·80 | 91·5 | 8·5 |
| 7·40 | 80·8 | 19·2 | | | |

Whilst the phosphate solutions will keep in the refrigerator in hard glass vessels for a few weeks it is advisable to make up the *p*H standards freshly on the day on which they are to be used.

**Technique.** Set up four tubes of similar bore as follows :

1. Twenty ml. of saline-phenol red solution covered with oil, into which 1 ml. of plasma is run, preferably from an Ostwald pipette with marks above and below the bulb.
2. Twenty ml. of 0·9 per cent. saline under oil plus 1 ml. of plasma.
3. The standard *p*H tube.
4. Water.

Arrange the four tubes in a comparator as shown (Fig 62) so that 1 and 4 are compared with 2 and 3. Find which *p*H tube gives the nearest match. The *p*H is thus obtained to the nearest 0·05 or even to the nearest 0·025.

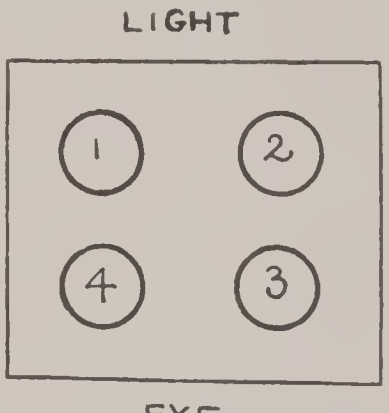


Fig. 62. Comparator.

Cullen corrected for the difference in temperature between that of the body and that at which the test is carried out.

*p*H at 38° C. = *p*H measured by colorimetric method — (0·42 — 0·01 *t*) where *t* = temperature in ° C. of the test. This is reasonably satisfactory for normal human plasma but varies with some pathological plasmas.

The plasma must be separated immediately from blood taken under oil and must be kept constantly under oil. Hawkins modified the method so as to use small amounts of whole blood. He placed 5 ml. of the saline-phenol red solution under paraffin in a test tube and drew the blood directly into a 1 ml. pipette graduated in 0·01 ml. by attaching a needle with a short rubber to the pipette. After detaching the needle and rubber he ran 0·25 ml. under oil into the tube containing the saline-phenol red solution and mixed by stirring carefully with a clean glass rod. The same amount of blood was run under oil into a tube containing 5 ml. of 0·9 per cent. saline and mixed. The two tubes were centrifuged and compared as above using two further tubes containing the appropriate phosphate standard and water respectively.

INTERPRETATION

The normal range for plasma *p*H is from 7·3 to 7·5. The *p*H remains constant within these limits during the earlier stages of an acidosis or an alkalosis because of the operation of compensatory processes. So *p*H may still be within normal limits when the alkali reserve is moderately raised or lowered. In such cases its only value would be to show that compensation was adequate up to that point. When the body is unable to compensate, *p*H becomes abnormal, falling below 7·3 in acidosis and rising above 7·5 in alkalosis. The extreme range in disease is from about 6·95 to 7·8.

## Determination of Urinary Ammonia

Formation of ammonia by the kidneys is a recognized method by which the body removes acids, excreting them as their ammonium salts. Two methods of estimating such ammonium salts will be described.

1. **Aeration-titration.** The apparatus used is that for the estimation of blood urea by the urease aeration method (p. 106). Measure 5 ml. of urine into a cylinder and connect up to a second cylinder containing 50 ml. of N/100 sulphuric acid. Add 5 grams of potassium carbonate and 5 ml. of saturated potassium carbonate solution to the urine. Aerate slowly at first and then more quickly for about twenty minutes. Then withdraw 25 ml. of the acid and titrate with N/100 sodium hydroxide. Titrate 25 ml. of the acid with the alkali. This last should be very nearly 25 ml.

**Calculation.** If T is the titration of the test, that of the blank, and V the volume of urine used, then

Grams ammonia per litre of urine

$$= \frac{(B - T)}{V} \times 0{\cdot}34 \text{ (as } NH_3\text{) or } \frac{(B - T)}{V} \times 0{\cdot}28 \text{ (as N).}$$

So with 5 ml. urine

Grams ammonia per litre of urine

$= (B - T) \times 0{\cdot}068$ as $NH_3$ or $\times$ 0·056 as N.

2. **Nesslerization after Adsorption with Permutit.** Folin and Bell, 1917.

**Reagents.** 1. Permutit.

2. Sodium hydroxide, 10 per cent. solution.

3. Standard ammonia solution containing 10 mg. ammonia per 100 ml. Dissolve 0·388 gram ammonium sulphate in 1 litre of solution.

4. Nessler's solution (see p. 104).

**Technique.** Place about 2 grams of permutit and about 5 ml. of distilled water into a 200 ml. volumetric flask. Then add 2 ml. of urine. From 1 ml. to 5 ml. may be used depending on the amount of ammonia present, but it is better not to use more than 2 ml. Wash in the added urine if necessary with up to 5 ml. of water. Shake the mixture gently for about five minutes. Add 25 to 40 ml. of water, mix gently, allow to settle and decant the supernatant fluid. Repeat this addition of water and decanting. Two additional

washings may be needed if bile is present. After these washings add a little water followed by 5 ml. of the 10 per cent. sodium hydroxide. Mix and then fill the flask three-quarters-full with water. Shake and add 10 ml. of Nessler's reagent. Mix and allow to stand for ten minutes. Then make to the mark, mix, allow to settle and compare the supernatant fluid with a standard prepared by adding to 5 or 10 ml. of the above solution of ammonium sulphate in a 200 ml. volumetric flask, 5 ml. of 10 per cent. sodium hydroxide, water to three-quarters full, 10 ml. of Nessler's solution. Then make to the mark with water.

**Calculation.** If V = volume of urine used and $m$ = mg. of ammonia in the standard solution, then if the photoelectric colorimeter is used,

Grams of ammonia per litre urine

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{m}{V}.$$

**Notes.** 1. Care has to be taken to see that ammonia is neither lost from the urine, since ammonia may escape from alkaline urine, nor formed by the action of bacteria on substances such as urea. If the urine is made acid by addition of hydrochloric acid these changes may be prevented and the urine kept in a refrigerator. But prolonged storage is not desirable. The estimation is best done soon after collection into a few ml. of acid if only ammonia is to be determined. If titrations of urinary acidity are to be done (see below) this cannot be used and the urine should be tested shortly after being passed. Otherwise collect into a clean bottle, add toluene, and place in a refrigerator, but do not keep more than twenty-four hours.

2. Folin and Bell recommend permutit which will pass through a 60-mesh but not through an 80 mesh sieve. Test out the activity on the standard ammonia solution used. If, after shaking with water, the solid does not separate out leaving a clear solution, shake and decant this turbid solution which does not settle.

The permutit can be used over and over again. After freeing from ammonia with 10 per cent. sodium hydroxide, wash with 2 per cent. acetic acid, decant, wash with water, decant, and dry in air without heat. The solid powder so obtained contains about 20 per cent. of combined water, which if driven off, leaves a powder with its activity greatly reduced.

INTERPRETATION

The normal daily output of ammonia is about 0·7 gram on an average diet. The ammonia nitrogen forms from 2·5 to 4·5 per cent. of the total urinary non-protein nitrogen. This percentage is termed the *ammonia coefficient*. It varies greatly. There is an increase when acid-forming foods are taken and a decrease with alkali-producing ones. There is a marked increase in the acidosis of diabetes when up to 5 grams and over may be excreted, forming as much as 40 per cent. of the total urinary non-protein nitrogen. There is also

an increase in the acidosis of starvation. Formation of ammonia by the kidneys is one of the methods by which the body removes acids without losing base. However, this does not operate in advanced renal disease so that there is not the same increase in urinary ammonia in the acidosis then present.

### Titratable Acidity of Urine

This term is given to the volume of N/10 sodium hydroxide required to make the urine alkaline to phenolphthalein (Folin, 1903), or to titrate it to *p*H 7·4, which is the *p*H of blood (Henderson and Palmer, 1914). In the method of Folin a twenty-four-hour specimen is collected, and 5 grams of powdered potassium oxalate mixed with 25 ml. of the urine, which is then titrated with N/10 sodium hydroxide, using 2 drops of 1 per cent. phenolphthalein as indicator, until a permanent pink colour is reached.

Titratable acidity

$$= \text{ml. N/10 sodium hydroxide} \times \frac{\text{volume of twenty-four-hour urine}}{25}$$

Henderson and Palmer titrated 10 ml. of urine and used neutral red as indicator (0·2 ml. of 2 per cent. solution in water). Phenol red can be used, for example that used in the titration method for plasma *p*H. Ten ml. of a phosphate buffer with this indicator added is employed as standard.

The chief effect of the titration is to convert dihydrogen phosphate into monohydrogen phosphate. Organic acids are also titrated. Thus β-hydroxybutyric acid in diabetic acidosis, the only one likely to be present in considerable amount, may be about 50 per cent. free at *p*H 5·0, whereas at *p*H 7·4 it is wholly present as salts.

By the Folin method the titratable acidity is 200 to 500 ml. N/10 sodium hydroxide per twenty-four hours. The Henderson-Palmer method naturally gives lower figures, the range being 150 to 400. Larger volumes are required in acidosis, smaller ones in alkalosis. Values up to 1,500 are found in severe diabetic acidosis.

The sum of the titratable acidity and the ammonia excretion gives a measure of the extent to which the body is able to conserve base. Gamble *et al.* (1923) used the term base economy for this less the plasma bicarbonate.

### *p*H of Urine

It is sometimes useful to know the *p*H of the urine. Hastings *et al.*, (1925) used a method similar to that for plasma, adding indicator to the urine and comparing with standards. For ordinary routine work several simple methods are available based on this principle. Thus British Drug Houses have sets of capillator standards for several *p*H ranges. Special indicators are issued for these so that equal volumes of urine and indicator measured in a capillary tube are mixed and compared with the standards. Suitable sets for urine are :

| | |
|---|---|
| Methyl red | *p*H 4·4 to 6·0 |
| Brom cresol purple | *p*H 5·2 to 6·8 |
| Brom thymol blue | *p*H 6·0 to 7·6 |
| Phenol red | *p*H 6·8 to 8·4 |

In addition the same company have booklets of indicator papers suitable for this purpose, those covering the range for urine *p*H being from *p*H 4·0 to 5·5 ; 5·5 to 7·0 ; and 7·0 to 8·5.

Nitrazine indicator papers are also suitable for use with urines.

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## *CHAPTER XX*

# BASAL METABOLISM. OXYGEN CAPACITY

By basal metabolism we understand the amount of energy required just to maintain the body processes when a person is at complete physical and mental rest and is some twelve to fourteen hours from the time when food was last taken. Such standard or basal conditions afford a sufficiently reproducible and reliable means of comparing metabolism in health and disease. They do not, however, correspond to the smallest energy needs of the body, since in sleep, energy requirements may sometimes fall below those of the basal metabolism as measured under the above conditions.

As with other energy requirements, basal metabolism is expressed in terms of *calories*. These are the calories used in nutrition and are the large calories of physics, one such calorie being the energy required to raise the temperature of 1 kilogram of water 1° C. Basal metabolism, however, may also be expressed directly in terms of the oxygen consumption under the same conditions.

### Factors influencing Basal Metabolism

Basal metabolism in normal persons varies with size and with differences in the rate at which the body processes are taking place. It is naturally greater as both these increase, so that in drawing up normal standards both these factors have to be allowed for.

Variations in size are considered to be more accurately referred to changes in surface area than to changes in weight and are held to be proportional to the surface area, not to the weight. Surface area diminishes less rapidly than does weight, so that a person such as a child with half the weight of an adult has more than half the surface area of that adult. So the requirements of basal metabolism for children, and for small persons generally, are greater per unit weight than those of larger persons, since their surface area per unit weight is greater. There are also some variations in surface area among people with roughly similar weight, since tall, thin persons have greater surface area than have shorter, stouter individuals of the same weight. As a result the basal metabolism of the former will tend to be higher per unit weight than that of the latter.

In the second place, the basal metabolism varies with the intensity at which the body processes are occurring. This, for example, is a little greater in men than in women, so that their basal metabolism per unit surface area is greater by about 7 per cent. Then, again, the rate of metabolism is appreciably greater in the case of children than in adults so that in young children it may be greater by as much as 25 per cent. per unit surface area. This, together with their greater relative surface area for their weight, makes the basal metabolism of children per unit weight considerably higher than that of adults.

Finally, it should be noted that in older people the rate of metabolism falls when compared with that found in adults between twenty and forty years old, so that it may be as much as 20 per cent. lower by the time the seventies are reached.

These differences are reflected in the figures given in the tables. Table XX gives the basal metabolism in calories per square metre of surface area per hour for different ages, the ages being taken to the nearest whole year. These are the Aub-DuBois standards as modified by Boothby and Sandiford (1929) which have been most widely used.

TABLE XX

*Standards for Basal Metabolism* (Boothby and Sandiford)

In calories per square metre per hour

| Age | Males | Females | Age | Males | Females |
|---|---|---|---|---|---|
| 5 | (53·0) | (51·6) | 20–24 | 41·0 | 36·9 |
| 6 | 52·7 | 50·7 | 25–29 | 40·3 | 36·6 |
| 7 | 52·0 | 49·3 | | | |
| 8 | 51·2 | 48·1 | 30–34 | 39·8 | 36·2 |
| 9 | 50·4 | 46·9 | 35–39 | 39·2 | 35·8 |
| 10 | 49·5 | 45·8 | 40–44 | 38·3 | 35·3 |
| 11 | 48·6 | 44·6 | 45–49 | 37·8 | 35·0 |
| 12 | 47·8 | 43·4 | | | |
| 13 | 47·1 | 42·0 | 50–54 | 37·2 | 34·5 |
| 14 | 46·2 | 41·0 | 55–59 | 36·6 | 34·1 |
| 15 | 45·3 | 39·6 | 60–64 | 36·0 | 33·8 |
| 16 | 44·7 | 38·5 | 65–69 | 35·3 | 33·4 |
| 17 | 43·7 | 37·4 | | | |
| 18 | 42·9 | 37·3 | 70–74 | (34·8) | (32·8) |
| 19 | 42·1 | 37·2 | 75–79 | (34·2) | (32·3) |

Boothby *et al.*, (1936) have published further figures for the basal metabolism of normal individuals which do not, however, differ materially from those given above.

Recently Robertson and Reid (1952) have published a set of standards the result of twenty years' work on normal persons in the London area. These are given in Table XXI and show the mean output per square metre per hour for different ages for both sexes and also the limits between which 95 per cent. of normal persons lie.

These results are lower than the Boothby and Sandiford standards for all ages except the youngest. This appears to be due to the fact that whereas Robertson and Reid used the method outlined subsequently (p. 384) in which the measurement is repeated until consistent results are obtained, Boothby *et al.*, took " the first determination made for the individual unless at the time of the test and before its calculation it was noted as unsatisfactory for reasons of restlessness,

## Table XXI

*Standards for Basal Metabolism* (Robertson and Reid)

Expected Mean Heat Output and Limits of Variation (Calories per square metre body-surface per hour)

| Age last birthday (year) | Males | | | Females | | |
|---|---|---|---|---|---|---|
| | Expected mean | Lower limit | Upper limit | Expected mean | Lower limit | Upper limit |
| 3 | 60·1 | 51·5 | 68·8 | 54·5 | 46·4 | 62·6 |
| 4 | 57·9 | 49·2 | 66·5 | 53·9 | 45·8 | 62·1 |
| 5 | 56·3 | 47·7 | 65·0 | 53·0 | 44·9 | 61·1 |
| 6 | 54·2 | 45·6 | 62·8 | 51·8 | 43·6 | 59·9 |
| 7 | 52·1 | 43·4 | 60·7 | 50·2 | 42·1 | 58·3 |
| 8 | 50·1 | 41·4 | 58·7 | 48·4 | 40·2 | 56·5 |
| 9 | 48·2 | 39·6 | 56·7 | 46·4 | 38·2 | 54·5 |
| 10 | 46·6 | 38·6 | 54·5 | 44·3 | 36·3 | 52·5 |
| 11 | 45·1 | 37·7 | 52·2 | 42·4 | 35·1 | 50·6 |
| 12 | 43·8 | 36·8 | 50·4 | 40·6 | 34·0 | 47·7 |
| 13 | 42·7 | 36·0 | 49·3 | 39·1 | 33·2 | 45·1 |
| 14 | 41·8 | 35·2 | 48·4 | 37·8 | 32·6 | 42·7 |
| 15 | 41·0 | 34·4 | 47·6 | 36·8 | 32·2 | 41·7 |
| 16 | 40·3 | 33·7 | 46·9 | 36·0 | 31·3 | 40·8 |
| 17 | 39·7 | 33·1 | 46·3 | 35·3 | 30·7 | 40·2 |
| 18 | 39·2 | 32·9 | 45·6 | 34·9 | 30·1 | 39·7 |
| 19 | 38·8 | 32·7 | 45·0 | 34·5 | 29·7 | 39·3 |
| 20 | 38·4 | 32·6 | 44·3 | 34·3 | 29·5 | 39·1 |
| 21 | 38·1 | 32·5 | 43·7 | 34·1 | 29·3 | 38·9 |
| 22 | 37·8 | 32·4 | 43·1 | 34·0 | 29·2 | 38·8 |
| 23 | 37·6 | 32·3 | 42·6 | 34·0 | 29·1 | 38·8 |
| 24 | 37·3 | 32·2 | 42·3 | 33·9 | 29·1 | 38·8 |
| 25 | 37·1 | 32·1 | 42·1 | 34·0 | 29·1 | 38·8 |
| 26 | 37·0 | 32·0 | 42·0 | 34·0 | 29·2 | 38·8 |
| 27 | 36·8 | 31·8 | 41·8 | 34·0 | 29·2 | 38·8 |
| 28 | 36·6 | 31·6 | 41·6 | 34·0 | 29·2 | 38·9 |
| 29 | 36·5 | 31·5 | 41·5 | 34·1 | 29·2 | 38·9 |
| 30 | 36·4 | 31·4 | 41·4 | 34·1 | 29·2 | 38·9 |
| 31 | 36·3 | 31·3 | 41·3 | 34·0 | 29·2 | 38·8 |
| 32 | 36·2 | 31·2 | 41·2 | 33·9 | 29·1 | 38·8 |
| 33 | 36·1 | 31·1 | 41·1 | 33·8 | 29·0 | 38·7 |
| 34 | 36·0 | 31·0 | 41·0 | 33·7 | 28·9 | 38·5 |
| 35 | 35·9 | 30·9 | 40·9 | 33·5 | 28·7 | 38·3 |
| 36 | 35·8 | 30·8 | 40·8 | 33·3 | 28·5 | 38·2 |
| 37 | 35·7 | 30·7 | 40·7 | 33·1 | 28·3 | 38·0 |
| 38 | 35·7 | 30·7 | 40·7 | 32·9 | 28·0 | 37·7 |
| 39 | 35·6 | 30·6 | 40·6 | 32·8 | 28·0 | 37·6 |
| 40 | 35·5 | 30·5 | 40·5 | 32·6 | 27·8 | 37·5 |

TABLE XXI—*continued*

| Age last birthday (year) | Males | | | Females | | |
|---|---|---|---|---|---|---|
| | Expected mean | Lower limit | Upper limit | Expected mean | Lower limit | Upper limit |
| 41–44 | 34·5 | 29·5 | 39·5 | 32·5 | 27·7 | 37·4 |
| 45–49 | 34·1 | 29·1 | 39·1 | 32·2 | 27·4 | 37·1 |
| 50–54 | 33·8 | 28·8 | 38·8 | 31·9 | 27·1 | 36·7 |
| 55–59 | 33·4 | 28·4 | 38·4 | 31·6 | 26·8 | 36·4 |
| 60–64 | 33·1 | 28·1 | 38·1 | 31·3 | 26·5 | 36·1 |
| 65–69 | 32·7 | 27·7 | 37·7 | 31·0 | 26·2 | 35·8 |
| 70–74 | 32·4 | 27·4 | 37·4 | 30·7 | 25·9 | 35·5 |
| 75 or more | 32·0 | 27·0 | 37·0 | — | — | — |

observable nervous tension, or an elevated temperature." The element of training was thus deliberately excluded. However, as Robertson points out, if successive determinations are to be made, this is not possible, and also, so-called nervous tension is not uncommon when the test is first done, and is specially likely to occur in thyrotoxicosis, so that initial readings may be 10 to 20 per cent. and more higher than those finally obtained. It appears that Robertson's technique is to be preferred and his standards used.

The question of standards is also discussed by Dubois (1936), and by Peters and Van Slyke (1932, 1946). The basal metabolism of children and adolescents has been specially studied by a number of workers (see, for example, Talbot, 1925, Lewis *et al.*, 1937, for children, and Shock, 1942, for a study of adolescents). There is general agreement

TABLE XXII

*Standards for Basal Metabolism in Childhood and Adolescence* (Lewis *et al.*, and Shock)

(In calories per square metre per hour)

| Age | Males (Lewis *et al.*) | Females (Lewis *et al.*) | Age | Males (Shock) | Females (Shock) |
|---|---|---|---|---|---|
| 2 | 54·3 | 52·6 | 11·5 | 43·6 | 41·7 |
| 3 | 53·1 | 51·2 | 12·0 | 45·0 | 41·0 |
| 4 | 51·9 | 49·8 | 12·5 | 44·4 | 40·4 |
| 5 | 50·7 | 48·5 | 13·0 | 44·1 | 39·9 |
| 6 | 49·5 | 47·1 | 13·5 | 43·2 | 38·8 |
| 7 | 47·1 | 45·7 | 14·0 | 43·5 | 38·0 |
| 8 | 45·9 | 44·3 | 14·5 | 41·4 | 36·5 |
| 9 | 44·7 | 43·0 | 15·0 | 42·8 | 35·7 |
| 10 | 43·5 | 41·6 | 15·5 | 41·4 | 34·4 |
| 11 | 42·5 | 40·2 | 16·0 | 41·1 | 34·2 |
| — | — | — | 16·5 | 41·0 | 34·6 |

that just after birth the basal metabolism is low and that it rises during the first year or two of life to reach a peak, after which it slowly declines. The age at which the maximum occurs has been rather variously placed about two to three years, but from the work of Lewis *et al.*, it appears to occur before two years of age. Some workers have held that there is a rise in basal metabolism during puberty.

The standards for basal metabolism during childhood and adolescence given by Lewis *et al.*, and Shock and shown in Table XXII, are appreciably lower than those of Boothby and Sandiford and in some cases even a little lower than those of Robertson and Reid.

### Measurement of Basal Metabolism

Both direct and indirect calorimetry can be used for measuring basal metabolism.

**Direct Calorimetry.** In this we measure the actual heat production using a respiration chamber, of which only a few exist. Because of the elaborate apparatus required, this method is not used in routine work. Details are given in larger works on metabolism and nutrition.

**Indirect Calorimetry.** In this we measure the volume of oxygen consumed, or of the oxygen consumed and the carbon dioxide produced, over an accurately timed period of a few minutes. From these the energy utilized can be calculated.

The volume of oxygen used by the body varies to some extent with the type of food metabolized. Thus, when carbohydrate is being used, the consumption of 1 litre of oxygen represents the production of 5·05 calories. For fat and proteins the corresponding figures are 4·69 and 4·49 respectively. On an average mixed diet the consumption of 1 litre of oxygen represents the production of 4·80 calories. If we ignore the nature of the diet and use this figure to calculate the calorie production from the volume of oxygen consumed per minute, the maximum error introduced will be either − 5 per cent or + 6 per cent. In most cases it will be much smaller. Instead of converting the ml. of oxygen consumed per minute into calories, tables can be drawn up giving the normal oxygen consumption for different heights and weights. The observed oxygen consumption in a particular case is compared with this normal and the result then reported as a percentage increase or decrease. This method is used in closed circuit methods of estimating basal metabolism. The patient breathes in air or oxygen from a reservoir to which the expired air is returned through soda lime to absorb the carbon dioxide produced. The oxygen consumed is read directly as the decrease in volume of the gas in the reservoir.

For more accurate work it is necessary to take into account the type of nutrients metabolized. To do this, both the oxygen consumed and the carbon dioxide produced are measured, and the *respiratory quotient* calculated.

$$\text{Respiratory quotient, R.Q.} = \frac{\text{Volume of carbon dioxide produced}}{\text{Volume of oxygen consumed}}$$

in a given period. This differs for the different nutrients. Thus, when carbohydrates are metabolized, the process may be represented

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6\ CO_2 + 6\ H_2O$$

so that the respiratory quotient is exactly 1·00.

Fats contain a much greater proportion of hydrogen to oxygen than do carbohydrates, and so produce more water. Taking tripalmitin as example, we have

$$2\ C_{51}H_{98}O_6 + 145\ O_2 \rightarrow 102\ CO_2 + 98\ H_2O$$

giving a respiratory quotient of 0·7. This varies a little for different fats. For the mixture present in the human diet it is taken as 0·707.

The case of proteins is more complicated since they are not completely metabolized in the body. Most of the nitrogen, for example, is excreted as urea in the urine. However, it is found that the respiratory quotient averages 0·80.

On an ordinary mixed diet the respiratory quotient is about 0·85. When the body is in the post-absorptive state it is about 0·82. It is clear that the respiratory quotient gives some indication of the type of food being consumed. When carbohydrate only is metabolized, the quotient is 1·00 and the calorie equivalent of a litre of oxygen is 5·05. If only fat is being metabolized the R.Q. is 0·707 and the calorie equivalent of a litre of oxygen is 4·69. It is thus possible to draw up a table giving the calorie equivalent for the range of respiratory quotients from 0·70 to 1·00 for all mixtures of fat and carbohydrate. This respiratory quotient is termed the non-protein R.Q. Such a table is given, Table XXIII.

TABLE XXIII

*Respiratory Quotients for Different Mixtures of Fat and Carbohydrate*

(Lusk, 1924)

| R.Q. | Calories per litre $O_2$ | Per cent. total oxygen consumed by | | R.Q. | Calories per litre $O_2$ | Per cent. total oxygen consumed by | |
|---|---|---|---|---|---|---|---|
| | | Carbohydrate | Fat | | | Carbohydrate | Fat |
| 0·707 | 4·686 | 0 | 100 | 0·86 | 4·88 | 52·2 | 47·8 |
| 0·71 | 4·69 | 1·02 | 99·0 | 0·87 | 4·89 | 55·6 | 44·4 |
| 0·72 | 4·70 | 4·44 | 95·6 | 0·88 | 4·90 | 59·0 | 41·0 |
| 0·73 | 4·71 | 7·85 | 92·2 | 0·89 | 4·91 | 62·5 | 37·5 |
| 0·74 | 4·73 | 11·3 | 88·7 | 0·90 | 4·92 | 65·9 | 34·1 |
| 0·75 | 4·74 | 14·7 | 85·3 | 0·91 | 4·94 | 69·3 | 30·7 |
| 0·76 | 4·75 | 18·1 | 81·9 | 0·92 | 4·95 | 72·7 | 27·3 |
| 0·77 | 4·76 | 21·5 | 78·5 | 0·93 | 4·96 | 76·1 | 23·9 |
| 0·78 | 4·78 | 24·9 | 75·1 | 0·94 | 4·97 | 79·5 | 20·5 |
| 0·79 | 4·79 | 28·3 | 71·7 | 0·95 | 4·99 | 82·9 | 17·1 |
| 0·80 | 4·80 | 31·7 | 68·3 | 0·96 | 5·00 | 86·3 | 13·7 |
| 0·81 | 4·81 | 35·2 | 64·8 | 0·97 | 5·01 | 89·8 | 10·2 |
| 0·82 | 4·83 | 38·6 | 61·4 | 0·98 | 5·02 | 93·2 | 6·8 |
| 0·83 | 4·84 | 42·0 | 58·0 | 0·99 | 5·04 | 96·6 | 3·4 |
| 0·84 | 4·85 | 45·4 | 54·6 | 1·00 | 5·05 | 100·0 | 0 |
| 0·85 | 4·86 | 48·8 | 51·2 | | | | |

The non-protein R.Q. can be determined by measuring the oxygen

consumption, the carbon dioxide production, and the nitrogen excretion over a measured time. From the nitrogen excretion the amount of protein being metabolized is calculated

grams protein metabolized = grams urinary nitrogen × 6·25.

Metabolism of a gram of urinary nitrogen consumes 5·94 litres of oxygen and produces 4·76 litres of carbon dioxide. We can thus find the volume of oxygen consumed and the volume of carbon dioxide produced as a result of the protein metabolized. By subtracting these from the volumes found experimentally we can obtain the oxygen consumed and carbon dioxide produced by the metabolism of fat and carbohydrate. This gives us the non-protein respiratory quotient and from the table the caloric equivalent of the oxygen consumed in their metabolism. The caloric equivalent of the oxygen consumed in metabolizing protein is 4·485. The total energy utilization can then be calculated with as high a degree of accuracy as the collection of the expired air permits.

The amount of protein ordinarily metabolized is small compared with that of fat and carbohydrate. Only 10 to 12 per cent. of calories are derived from protein when ordinary mixed diets are being taken. In most determinations the effect of protein is ignored. The error thus introduced is small. The respiratory quotient is calculated from the carbon dioxide expired and the oxygen consumed. The calorie equivalent of a litre of oxygen is obtained from Table XXIII using this respiratory quotient as though it were the non-protein R.Q. The calories per square metre per hour can then be calculated and compared with the normal, and the percentage increase or decrease calculated.

This method of calculating the results can be used in the open circuit methods. In this the expired air is collected in a bag, and analysed for carbon dioxide and oxygen content in a gas analysis apparatus.

## Open Circuit Methods

In the method most commonly used, the patient breathes in ordinary atmospheric air and breathes out into a Douglas bag. This, shown in Fig. 63, is a rubber-lined bag which can hold up to a 100

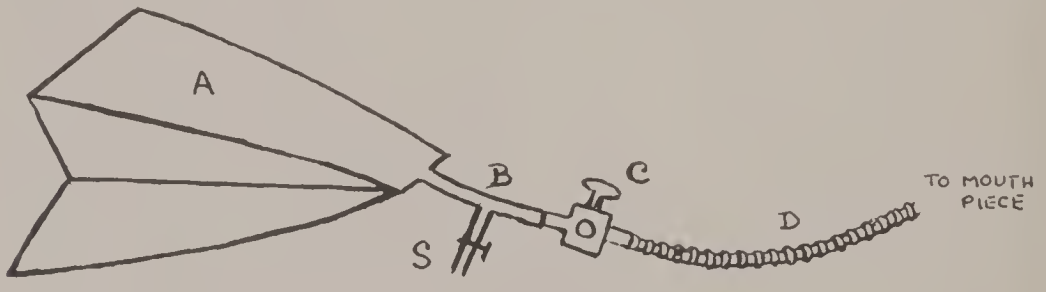


Fig. 63. Douglas Bag.

litres of air. The bag, A, has a short tube, B, attached to it, into the end of which is fitted a three-way tap, C. This allows the patient to breathe either directly into the bag or out into the atmosphere.

The other side of the tap is fitted with a length of flexible rubber tubing, D, about 1 inch in diameter which connects the bag with the device (Fig. 64) which carries the mouthpiece E which fits into the patient's mouth. This, shaped as shown, contains two valves, F and G, which consist of a flat rubber disc, *a*, and a perforated metal plate, *b*, arranged as shown. When the patient breathes in, air can enter through F but G closes since its rubber disc is drawn flat against the metal plate. On expiration the reverse takes place, for F closes and the expired air passes along the rubber tube into the bag or out into the atmosphere according to the way tap C is turned. Finally, it should be noted that the tube B between the tap and the bag is fitted with a narrow side tube, S, closed with a clip, through which a small amount of gas can be taken for analysis.

In carrying out the test, the patient, properly prepared, see p. 384, fits the mouthpiece and puts on a nose clip to stop any breathing

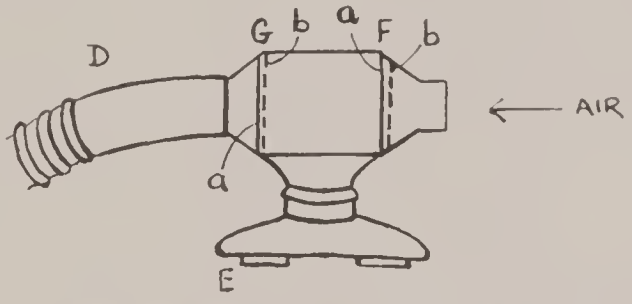


FIG. 64. Valves and Mouthpieces for use with Douglas Bag.

through the nose. Tap C is turned so that the expired air passes into the atmosphere. The Douglas bag should have been completely emptied. As soon as possible after the patient has fitted the nose clip, tap C is turned to allow the expired air to pass into the bag and a stop watch is started as this is done. After an accurately measured time, usually between five and ten minutes, tap C is again turned so that the patient breathes into the atmosphere, and the watch is stopped. The exact time is noted. Ten minutes is convenient but if the patient begins to show any signs of distress the test should be stopped at once. The bag and tubes should be arranged so that the patient is quite comfortable and is able to breathe normally. The tap can be turned on and off at the same point in the cycle of breathing though the error which results if this is not done is very small, being under 1 per cent.

A sample of the expired air is then analysed for oxygen and carbon dioxide content in a Haldane gas analysis apparatus. This is shown in Fig. 65. A gas sampler, A, is shown in position whereby a sample of gas from the bag can be introduced into the pipette B of the apparatus. This pipette, most conveniently of 10 ml. capacity, is calibrated from the top downwards so that the narrow part, *b*, represents from 7 to 10 ml. in 0·1 ml. Pipette B and control tube D are contained in a water bath E which can be agitated by blowing air through F. This serves to keep the gas in the pipette at a temperature which can be read accurately. The pipette containing the gas

is connected at its lower end with a thick rubber tube to the mercury reservoir M. The three-way taps C and G make it possible to connect B, first with a 10 per cent. sodium hydroxide solution in H, then with an alkaline pyrogallol solution (dissolve 10 grams of pyrogallol in 200 ml. of potassium hydroxide solution prepared by dissolving 160 grams in 130 ml. of water) in J. The former absorbs carbon dioxide, the latter oxygen. In N a sodium hydroxide solution is placed to seal off the rest of the apparatus from the atmosphere. The mercury reservoir, M, can be hooked at any convenient height in slots as shown. The apparatus is fastened to a vertical wooden board

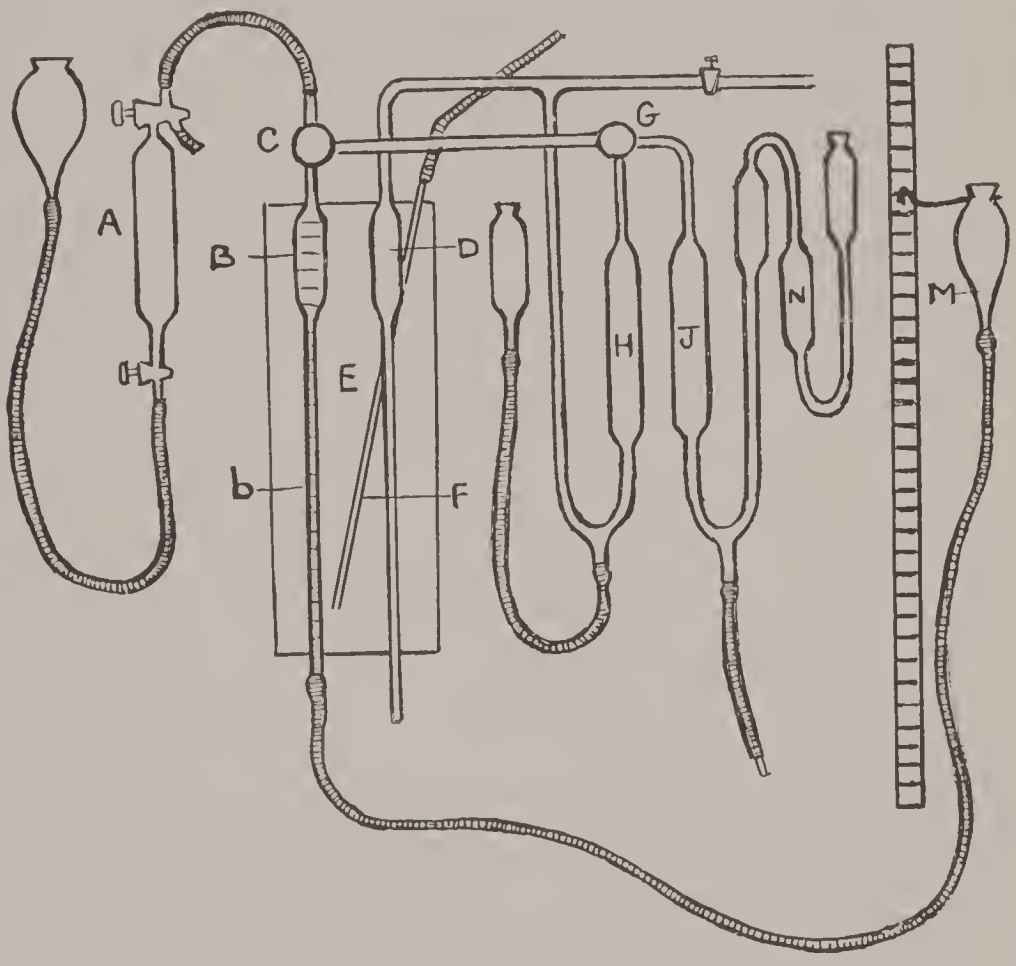


Fig. 65. Haldane Gas Analysis Apparatus.

supported in a wooden trough, which catches small amounts of mercury. The whole apparatus is compact and portable.

The pipette is almost filled with gas from the sampler by lowering M, and the volume measured with the mercury levels at the same height. The taps are then turned so as to connect the pipette with the caustic soda reservoir. The gas is driven in and out of the pipette by lifting the mercury up and down. When all the carbon dioxide has been absorbed, shown by the constant volume of the gas remaining, this is read with the two mercury levels at the same height and the tap G turned so as to connect with the alkaline pyrogallol. The oxygen is then absorbed in the same way so that

the further fall in volume gives the amount of oxygen present. It is then possible to calculate the percentage of carbon dioxide and oxygen in the expired air. The total volume of this expired air is measured by connecting the bag to a gas meter and forcing all the air through.

**Example of Calculation.** The data obtained are assumed to be

| | |
|---|---|
| Time of collection of expired air | 10 minutes. |
| Volume of expired air | 75 litres. |
| Barometric pressure | 750 mm. Hg. |
| Temperature | 16° C. |
| Expired air : Carbon dioxide | 3·6 per cent. |
| Oxygen | 16·75 per cent. |
| (By difference) Nitrogen | 79·65 per cent. |

Atmospheric air as breathed is taken to be : Carbon dioxide 0·04 per cent. ; Oxygen 20·93 per cent. ; Nitrogen 79·03 per cent.

The percentage of oxygen absorbed cannot be calculated by subtracting 20·93 — 16·75. The fact that there is more nitrogen

TABLE XXIV

*Correction of Oxygen Volumes*

Volume of Oxygen in Inspired Air corresponding to 100 ml. of Expired Air with different Percentages of Nitrogen

| Per cent. nitrogen in expired air | Oxygen in inspired air | Per cent. nitrogen in expired air | Oxygen in inspired air | Per cent. nitrogen in expired air | Oxygen in inspired air |
|---|---|---|---|---|---|
| 78·5 | 20·80 | 79·2 | 20·99 | 79·9 | 21·17 |
| 78·6 | 20·83 | 79·3 | 21·01 | 80·0 | 21·20 |
| 78·7 | 20·85 | 79·4 | 21·04 | 80·1 | 21·22 |
| 78·8 | 20·88 | 79·5 | 21·07 | 80·2 | 21·25 |
| 78·9 | 20·91 | 79·6 | 21·09 | 80·3 | 21·28 |
| 79·0 | 20·93 | 79·7 | 21·12 | 80·4 | 21·30 |
| 79·1 | 20·96 | 79·8 | 21·14 | 80·5 | 21·33 |

in the expired air than in atmospheric air shows that the volume of air breathed in must have been greater than that of the air breathed out. This is because part of the oxygen consumed was used to form water. The necessary correction can be made by multiplying the per cent. nitrogen in the expired air by 20·93/79·03, that is, by 0·265. This gives the volume of oxygen per 100 volumes of expired air. In the present case this is 79·65 × 0·265, which is 21·10, so the volume of oxygen used by the body is given by 21·10 — 16·75 = 4·35 volumes per cent. Table XXIV gives a list of such corrected oxygen volumes for the range of nitrogen percentages met.

It is necessary to correct the volume of gas to 760 mm. Hg. and 0° C. The pressure of water vapour has to be subtracted from the

TABLE XXV
*Water Vapour Pressure, mm. Hg.*

| °C. | mm. Hg. | °C. | mm. Hg. | °C. | mm. Hg. |
|---|---|---|---|---|---|
| 8 | 8·0 | 14 | 11·9 | 20 | 17·4 |
| 9 | 8·6 | 15 | 12·7 | 21 | 18·5 |
| 10 | 9·2 | 16 | 13·5 | 22 | 19·7 |
| 11 | 9·8 | 17 | 14·4 | 23 | 20·9 |
| 12 | 10·5 | 18 | 15·4 | 24 | 22·2 |
| 13 | 11·2 | 19 | 16·3 | 25 | 23·5 |

barometric pressure. At 16° C. this is 13·5 mm. Hg. (Table XXV).

Volume of expired air corrected to 0° C., 760 mm. Hg., is

$$75 \times \frac{750 - 13{\cdot}5}{760} \times \frac{273}{273 + 16} = 68{\cdot}7 \text{ litres.}$$

The oxygen consumption thus

$$= 68{,}700 \times \frac{4{\cdot}35}{100} \times \frac{1}{10} \text{ ml. per minute.}$$

$$= 299 \text{ ml. per minute.}$$

The patient was a male aged 26, weighing 11 stone and 5 feet 9 inches in height ; that is 154 lb. or 70 kilograms and 69 inches or 175 cm. Surface area is found to be 1·85 square metres (Table XXVI).

$$\text{Respiratory quotient} = \frac{3{\cdot}56}{4{\cdot}35} = 0{\cdot}82.$$

Ignoring proteins, at this R.Q. 4·82 calories correspond to each litre of oxygen. Hence

Basal metabolism in calories per square metre surface area per hour

$$= \frac{4{\cdot}82 \times \text{litres oxygen consumed per hour}}{\text{patient's surface area}}$$

$$= \frac{4{\cdot}82 \times 17{\cdot}94}{1{\cdot}84} = 47.$$

Normal value for this patient = 37·0 (Robertson and Reid).

Basal metabolism is thus increased by $\frac{10}{37} \times 100 = 27$ per cent

## Closed Circuit Methods

The various forms of apparatus which are used for this method are all based on the original Benedict-Roth apparatus, shown diagrammatically in Fig. 66. The patient breathes a supply of oxygen contained in the reservoir A. As the patient breathes out and in the reservoir moves up and down. The circulation of oxygen is shown by the arrows. On its way to the mouthpiece the oxygen passes through a valve which shuts when the patient breathes out, so that the expired air is returned along the other tube and passes

## Table XXVI

Surfaee Area (in square metres) from Height and Weight

| HEIGHT IN CENTIMETRES | WEIGHT IN KILOGRAMS | | | | | | | | | | | | | | | | | HEIGHT IN INCHES |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 | 65 | 70 | 75 | 80 | 85 | 90 | 95 | 100 | 105 | |
| 190 | | | | | | 1·70 | 1·77 | 1·84 | 1·90 | 1·96 | 2·02 | 2·08 | 2·13 | 2·18 | 2·23 | 2·28 | 2·33 | 75 |
| 185 | | | | | 1·59 | 1·67 | 1·74 | 1·80 | 1·87 | 1·92 | 1·98 | 2·04 | 2·09 | 2·14 | 2·19 | 2·24 | 2·29 | 73 |
| 180 | | | | 1·49 | 1·56 | 1·64 | 1·70 | 1·77 | 1·83 | 1·89 | 1·94 | 2·00 | 2·05 | 2·10 | 2·15 | 2·19 | 2·24 | 71 |
| 175 | | | 1·38 | 1·46 | 1·53 | 1·60 | 1·67 | 1·73 | 1·79 | 1·85 | 1·90 | 1·96 | 2·01 | 2·06 | 2·10 | 2·15 | 2·20 | 69 |
| 170 | | 1·26 | 1·35 | 1·43 | 1·50 | 1·57 | 1·63 | 1·69 | 1·76 | 1·81 | 1·86 | 1·91 | 1·96 | 2·01 | 2·06 | 2·11 | 2·15 | 67 |
| 165 | 1·14 | 1·23 | 1·32 | 1·40 | 1·47 | 1·53 | 1·60 | 1·66 | 1·72 | 1·77 | 1·82 | 1·87 | 1·92 | 1·97 | 2·02 | 2·06 | 2·11 | 65 |
| 160 | 1·12 | 1·21 | 1·29 | 1·36 | 1·43 | 1·50 | 1·56 | 1·62 | 1·68 | 1·73 | 1·78 | 1·83 | 1·88 | 1·93 | 1·97 | 2·01 | | 63 |
| 155 | 1·09 | 1·18 | 1·26 | 1·33 | 1·40 | 1·47 | 1·53 | 1·58 | 1·64 | 1·69 | 1·74 | 1·79 | 1·84 | 1·88 | 1·93 | | | 61 |
| 150 | 1·07 | 1·15 | 1·23 | 1·30 | 1·37 | 1·43 | 1·49 | 1·55 | 1·60 | 1·65 | 1·70 | 1·75 | 1·79 | 1·84 | | | | 59 |
| 145 | 1·04 | 1·12 | 1·20 | 1·27 | 1·34 | 1·40 | 1·45 | 1·51 | 1·57 | 1·61 | 1·66 | 1·71 | 1·75 | | | | | 57 |
| 140 | 1·01 | 1·10 | 1·17 | 1·24 | 1·30 | 1·36 | 1·42 | 1·47 | 1·53 | 1·57 | 1·62 | 1·66 | | | | | | 55 |
| 135 | 0·99 | 1·07 | 1·14 | 1·21 | 1·27 | 1·32 | 1·38 | 1·43 | 1·49 | 1·53 | 1·58 | | | | | | | 53 |
| 130 | 0·96 | 1·04 | 1·11 | 1·17 | 1·23 | 1·29 | 1·34 | 1·39 | 1·45 | 1·49 | | | | | | | | 51 |
| 125 | 0·93 | 1·01 | 1·08 | 1·14 | 1·20 | 1·25 | 1·31 | 1·36 | 1·41 | | | | | | | | | 49 |
| 120 | 0·91 | 0·98 | 1·05 | 1·11 | 1·16 | 1·22 | 1·27 | 1·32 | | | | | | | | | | 47 |
| 115 | 0·88 | 0·95 | 1·02 | 1·07 | 1·13 | 1·18 | 1·23 | | | | | | | | | | | 45 |
| 110 | 0·85 | 0·92 | 0·98 | 1·04 | 1·09 | 1·14 | | | | | | | | | | | | 43 |
| 105 | 0·82 | 0·89 | 0·95 | 1·01 | 1·06 | | | | | | | | | | | | | 41 |
| | 55 | 66 | 77 | 88 | 99 | 110 | 121 | 132 | 143 | 154 | 165 | 176 | 187 | 198 | 209 | 220 | 231 | |
| | WEIGHT IN POUNDS | | | | | | | | | | | | | | | | | |

These figures are calculated from Dubois' formula : Surfaee area in square metres
= Height in cm.$^{0·725}$ × Weight in kg.$^{0·425}$ × 71·84 ÷ 10,000.
Values for heights and weights intermediate between those given can easily be assessed.

through soda lime to absorb the carbon dioxide before returning to the reservoir. The amount of oxygen in the reservoir thus falls steadily during the course of the experiment. The upper end of the reservoir is connected over a pulley with a weight W to which is attached a pen. This records the movements of the reservoir on a slowly rotating drum, D, on which a suitably calibrated paper is wrapped. The drum is timed to rotate so that the length of time of the experiment and the change in the volume of gas in the reservoir are recorded on the paper. Each movement of the pen up and down

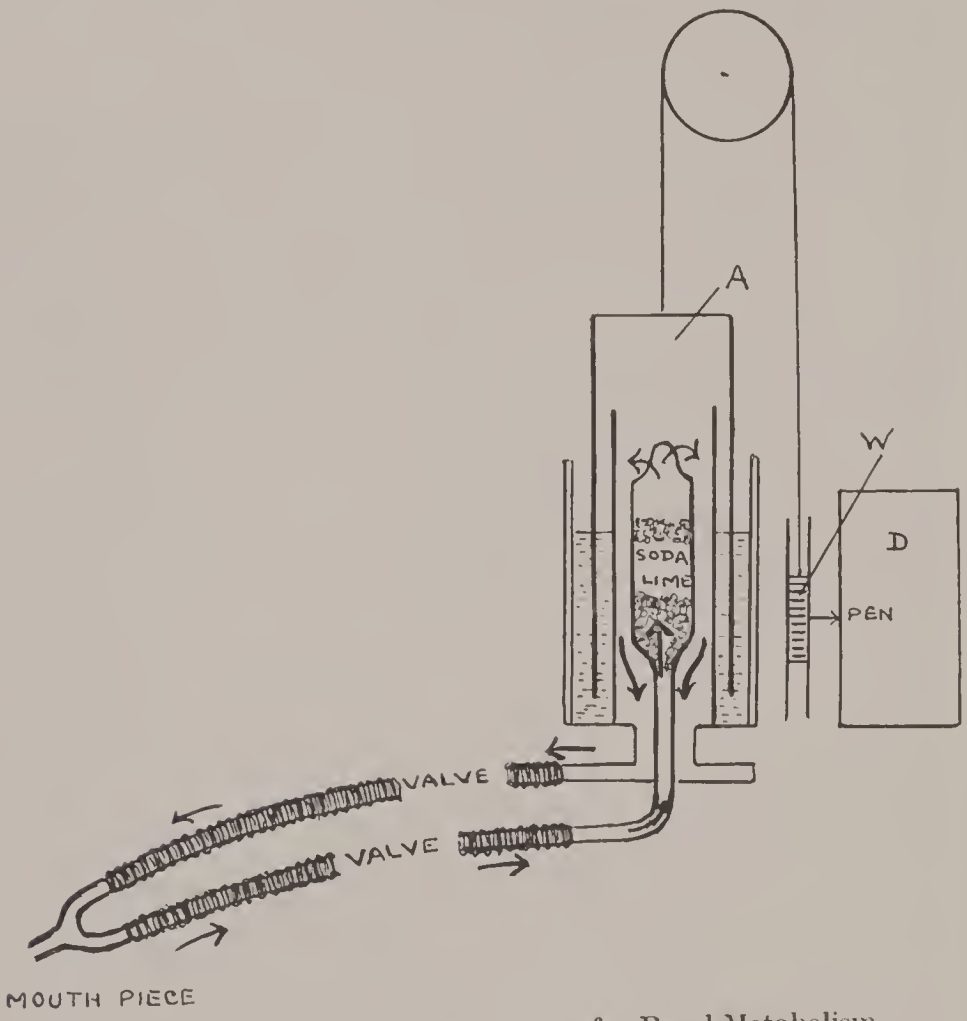


Fig. 66. Closed Circuit Apparatus for Basal Metabolism.

corresponds to a complete respiration, so that a line drawn along the crests or the troughs can be used to read the oxygen consumption. If such a line is to be drawn with sufficient accuracy, the size of the patient's respirations must be fairly uniform. In most cases it is possible to get the patient to breathe regularly enough. When this is not possible, an open circuit method has to be used.

The type of chart obtained is shown in Fig. 67. The volume of oxygen consumed per minute is easily calculated, and is then corrected to 0° C. and 760 mm. Hg. pressure. Variations in respiratory quotient are ignored and the consumption of a litre of oxygen corresponds to 4·82 calories. Hence the calories per square metre

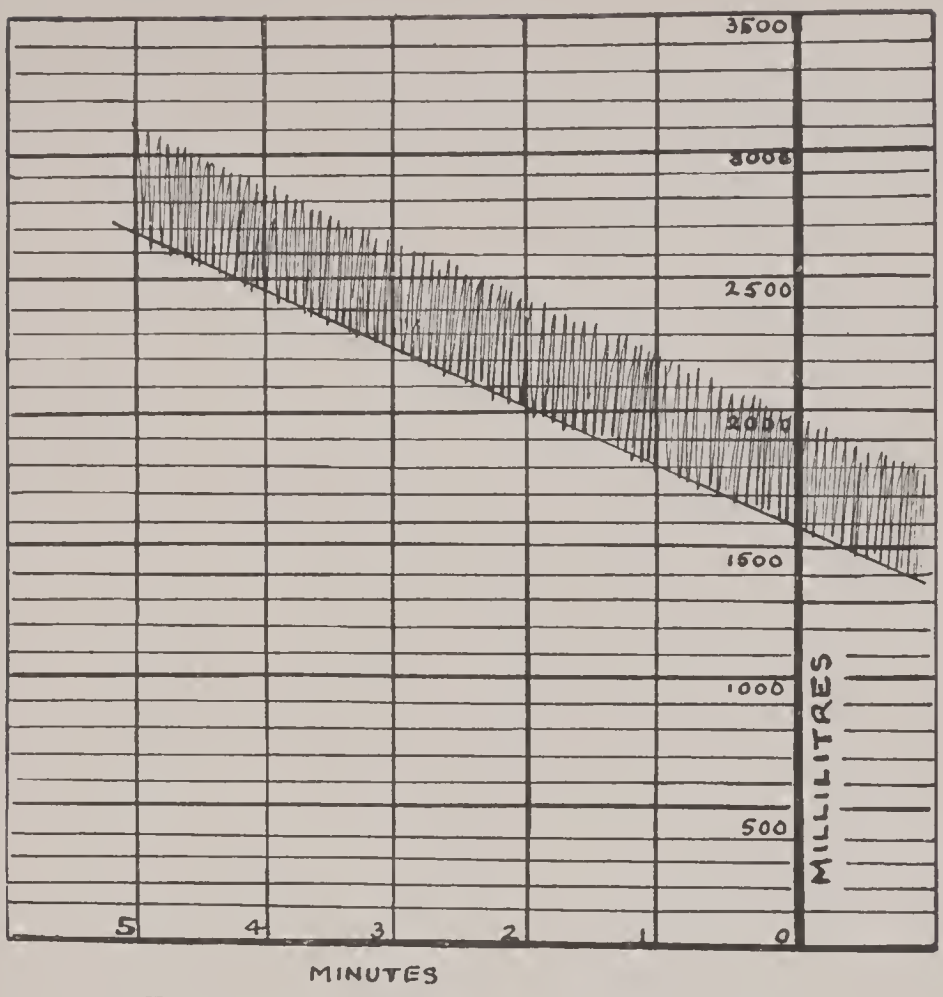


FIG. 67. Chart from Closed Circuit Apparatus.

## TABLE XXVII

*Factors for Converting the Volume of Gas to 0° C., 760 mm. Hg.*

| Pressure mm.Hg. | Temperature | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 8° | 10° | 12° | 14° | 16° | 18° | 20° | 22° | 24° | 26° |
| 720 | 0·920 | 0·914 | 0·908 | 0·901 | 0·895 | 0·889 | 0·883 | 0·877 | 0·871 | 0·865 |
| 725 | 0·927 | 0·920 | 0·914 | 0·907 | 0·901 | 0·895 | 0·889 | 0·883 | 0·877 | 0·871 |
| 730 | 0·933 | 0·927 | 0·920 | 0·914 | 0·907 | 0·901 | 0·895 | 0·889 | 0·883 | 0·877 |
| 735 | 0·940 | 0·933 | 0·926 | 0·920 | 0·914 | 0·907 | 0·901 | 0·895 | 0·889 | 0·883 |
| 740 | 0·946 | 0·939 | 0·933 | 0·926 | 0·920 | 0·914 | 0·907 | 0·901 | 0·895 | 0·889 |
| 745 | 0·952 | 0·946 | 0·939 | 0·933 | 0·926 | 0·920 | 0·913 | 0·907 | 0·901 | 0·895 |
| 750 | 0·959 | 0·952 | 0·945 | 0·939 | 0·932 | 0·926 | 0·919 | 0·913 | 0·907 | 0·901 |
| 755 | 0·965 | 0·958 | 0·952 | 0·945 | 0·939 | 0·932 | 0·926 | 0·919 | 0·913 | 0·907 |
| 760 | 0·972 | 0·965 | 0·958 | 0·951 | 0·945 | 0·938 | 0·932 | 0·925 | 0·919 | 0·913 |
| 765 | 0·978 | 0·971 | 0·964 | 0·958 | 0·951 | 0·944 | 0·938 | 0·931 | 0·925 | 0·919 |
| 770 | 0·984 | 0·977 | 0·970 | 0·964 | 0·957 | 0·951 | 0·944 | 0·938 | 0·931 | 0·925 |

Figures for intermediate values of temperature and pressure can easily be assessed.

Read the barometer, subtract from this the aqueous vapour pressure, and use the pressure obtained to find the correction factor in the above table for the temperature at which the volume of gas was measured.

of the patient's surface area per hour can be calculated as previously (p. 380) and compared with the normal standards given.

This method is so much simpler and less time consuming than the open circuit method using the Douglas bag, that it has come to be used almost exclusively for routine determinations in hospitals.

Apparatus of this type is made by Messrs. Kendrick, Woodford Green, Essex, and by Messrs. Palmer (London) Ltd., 63A Effra Road, Brixton, London, S.W.2.

### Preparation of the Patient. Tests on Out-patients

Careful preparation of the patient is most important if reliable results are to be obtained. The patient must be in a state of complete rest, both physical and mental. Since food influences the rate of metabolism, sufficient time must have passed since food was taken. Twelve hours from the last meal is generally accepted as being satisfactory, so it is convenient to do the test in the early morning. Breakfast is then delayed until after the test. As regards physical rest, if there has not been any vigorous muscular activity, it is sufficient if the patient lies down for half an hour immediately before the test. With hospital patients this presents no difficulty. The test can either be done at the bedside or the patient taken in bed to a special room. With out-patients, the half-hour rest is satisfactory provided activity has been small. But since the effect of any strenuous exercise in raising the rate of metabolism may extend over several hours, there must be an element of doubt as regards the reliability of tests done on out-patients. Robertson (1944) made a careful study of this subject and concluded that reliable results can be obtained if determinations are made on successive days. He used a rest of half an hour lying on a couch and made duplicate measurements of ten-minutes duration each morning. His results on the second day agreed within 5 per cent. in the great majority of cases and were rather lower than those obtained on the first morning. In the few cases in which there was not such agreement further estimations were carried out. These findings have been confirmed by other workers. The procedure suggested is as follows. After thirty minutes' rest, lying down, on the first morning, make an estimation, and repeat after a further rest of thirty minutes. This second estimation is usually lower than the first. A third estimation is made, in the same way, on the second morning. If this agrees with the second test done on the first morning, the investigation can be stopped at this point. If it does not a further estimation is done thirty minutes later. In the author's experience it is rare for agreement not to be obtained by this time. Under such conditions sufficiently accurate estimations can be made on out-patients.

It is equally important that the patient should be in a state of mental rest. Disturbing influences should be excluded as far as possible. In some hospitals the estimation is made in a separate room, set apart for this purpose. This avoids some of the disturbing effects which may be present in a ward, but it may cause some apprehension. A few explanatory words to the patient on arrival should give the necessary reassurance. In any case, any such fears should disappear after the first determination. The advantages as

regards freedom from disturbance make such a special room desirable whenever possible.

The patient should not read or talk during the period of resting and should be quite comfortable so that movements arising from discomfort are not often made. The bladder should be emptied shortly before beginning the test so that any discomfort from a full bladder does not interfere.

INTERPRETATION

In the table giving the standards of Robertson and Reid the normal range is shown. For the Boothby and Sandiford standards a range of ± 15 per cent. from the figures given is usually allowed for normal persons.

A high calorie diet, particularly if it is rich in protein, raises basal metabolism whilst a low calorie diet, poor in protein, tends to lower it. So in malnutrition, and in diseases in which it is present, some reduction in basal metabolism may be found. Fever raises basal metabolism by approximately 7 per cent. for each ° F., or 13 per cent. for each ° C., above normal. There is an increase in pregnancy and during lactation, which may reach + 30 per cent.

Determination of basal metabolism is mainly used in thyroid disease. Marked increases are found in hyperthyroidism, corresponding roughly to the severity of the condition. Findings range from normal to + 100 per cent., though even higher figures have been reported. The disease is severe if basal metabolism is more than + 50 per cent., very severe if it is over + 75 per cent. In hyperthyroidism the estimation is used to assess the severity of the disease, to follow the exacerbations and remissions to which the disease is subject, and to observe the effect of treatment. The estimation may also help in diagnosis in the small proportion of cases in which clinical signs are not as definite as usual and yet in which there is a considerable increase in basal metabolic rate. The estimation should be carried out with particular care in these cases. They are apt to give results which may be 20 to 30 per cent. too high on a first measurement because of the degree of apprehension aroused. Special care must be taken with all cases of hyperthyroidism to get the patient familiar with the apparatus. Otherwise results will not be reliable and clinical signs will provide a safer index.

Basal metabolism is reduced in hypothyroidism (myxœdema). In the severest cases values of − 50 per cent. are obtained, but the common range is from − 15 to − 35 per cent. The estimation is used both in diagnosis and in following treatment.

Such marked changes in basal metabolism are not often found in other endocrine disorders. Small increases to + 30 per cent. are sometimes seen in the early stages of acromegaly, when hyperpituitarism is present, though the majority are normal. Later, if there is hypopituitarism a slight reduction may be observed. In Fröhlich's syndrome only a small reduction is found, but in Simmonds' disease values as low as − 40 per cent. are seen. While Cushing's syndrome may show moderate increase, there is usually a small decrease in Addison's disease.

Few other conditions show alteration in basal metabolism. Moderate increases have been reported in leukæmia, both myelogenous and lymphatic, and in polycythemia vera. Values from — 15 to — 35 per cent. are seen in the earlier phases of Type II nephritis being lowest when the œdema is most extensive. The increase in body weight due to the œdema artificially raises the figure for the normal basal metabolism in these cases, but it is not universally accepted that this fully accounts for the low readings. Very occasional increases have been reported in hypertension.

With the exception of those cases in which there is endocrine dysfunction, basal metabolism is normal in obesity. Because it is low in malnutrition and in starvation, it is low in diseases in which there is under-nutrition. Thus it may be low in anorexia nervosa, particularly in severer cases, so that this condition cannot be differentiated from hypopituitarism by this means.

Reduced values are also found in shock.

## OXYGEN CONTENT AND OXYGEN CAPACITY

The oxygen present in blood is almost entirely loosely combined with hæmoglobin, that in simple physical solution forming only 2 or 3 per cent. of the total. Several different terms and quantities are used in connection with the blood oxygen.

The *oxygen content* of blood refers to the sum of the oxygen combined with hæmoglobin and that physically dissolved, which is present in the circulating blood in either the veins or the arteries. It is expressed in ml. oxygen, corrected to N.T.P., per 100 ml. blood. The blood has to be collected under oil so as to avoid loss of gas, or further oxygenation by contact with the oxygen of the atmosphere. By using a small correction for the oxygen present in physical solution, the amount actually *combined with hæmoglobin* can be obtained.

By *oxygen capacity* we mean the amount of oxygen which the blood contains when it is fully oxygenated. No precautions are necessary in the collection of the blood since the first step in its determination is to mix the blood with air. This is also expressed as ml. oxygen per 100 ml. of blood. If the volume of oxygen in physical solution is subtracted from the oxygen capacity we get the *oxygen combining power*, the volume of oxygen which is combined with the hæmoglobin of the blood when this is fully saturated. Consequently this affords a measure of the amount of hæmoglobin present in the blood. There is no agreed figure for the amount of oxygen which can combine with 1 gram of hæmoglobin, but 1·34 ml. is commonly used.

The *oxygen saturation* is the percentage of the oxygen combining power represented by the oxygen actually combined with hæmoglobin. It is thus the ratio of the oxygen content less the oxygen in physical solution to the oxygen capacity less the oxygen in physical solution, multiplied by 100.

*Oxygen unsaturation* is the opposite of oxygen saturation, and can be obtained by subtracting the oxygen saturation from 100.

Of these, the oxygen content and oxygen capacity are determined,

the others being derived from them. The determinations may be carried out using the Van Slyke apparatus described for the estimation of alkali reserve. The method is the same for both, except that the specimen of blood is collected differently.

### Determination of Oxygen Capacity and Oxygen Content

The blood is mixed in the Van Slyke apparatus with an oxygen reagent containing saponin to lyse the cells, potassium ferricyanide to liberate the oxygen, and caprylic alcohol to prevent frothing. A mixture of oxygen, carbon dioxide and nitrogen is obtained, the carbon dioxide being absorbed by sodium hydroxide.

**Reagents.** 1. Oxygen reagent. Dissolve 3 grams of potassium ferricyanide and 3 grams of saponin in water, add 3 ml. of caprylic alcohol and make up to a litre.

2. Sodium hydroxide, 2 per cent. solution.

**Technique.** Mix the oxygen reagent thoroughly and measure 7·5 ml. into cup C of the Van Slyke apparatus (Fig. 59). By lowering the mercury reservoir, transfer this to the pipette and deaerate by producing a vacuum. For *oxygen capacity*, place the blood in a separating funnel of about 300 ml. capacity, taking care if the blood has been standing, to see that the cells are completely and evenly distributed throughout the specimen. Allow the blood to form a thin layer over the surface of the funnel and rotate to ensure complete aeration of the hæmoglobin. For *oxygen content* take the blood under paraffin and keep it so, mixing thoroughly just before doing the estimation.

In both cases now proceed as follows. After removing gas from the oxygen reagent in the pipette, force 6 ml. of this into the cup, and pipette 2 ml. of the blood by means of an Ostwald pipette. Place the tip of the pipette at the bottom of the cup under the liquid, and run the blood and all the reagent except 1 ml. into the pipette of the apparatus. Seal off the tap by placing a small amount of mercury in the bottom of the cup. Lower the mercury reservoir to produce a partial vacuum, and shake until a constant volume of gas is obtained in the calibrated part of the pipette. From five to ten minutes is usually required. Run the liquid into the side tube F, wash out cup C with distilled water and place in it a few ml. of 2 per cent. sodium hydroxide. By suitably placing the mercury reservoir, run in slowly 0·5 ml. of this solution, which should have been deaerated previously. Introduce some mercury into cup C and run a fine stream down. This takes the hydroxide with it, otherwise it would remain at the upper end of the pipette. Mix, run as much liquid as possible into F, and again read the volume of gas, having adjusted the level of mercury in the reservoir so that it is higher than that of the mercury in the pipette by one-thirteenth of the height of any liquid still in the pipette. This gas is a mixture of oxygen from the hæmoglobin and of oxygen and nitrogen in physical solution. Ascertain the temperature and the barometric pressure.

**Calculation.** If $t$ = temperature in °C. and B = barometric pressure in mm. of mercury, and V = volume of gas (oxygen +

nitrogen) in ml., then the corrected volume of gas to 0° C. and 760 mm. Hg.

$$= \frac{V\,(B - w)}{760\left(\frac{t}{273} + 1\right)} = \frac{V\,(B - w)}{760\,(1 + 0{\cdot}00367t)}$$

where $w$ = vapour tension of water.

So *oxygen capacity* in ml. oxygen per 100 ml. blood

$$= \frac{V\,(B - w)}{760\,(1 + 0{\cdot}00367t)} \times \frac{100}{2} - 1{\cdot}9$$

since 2 ml. of blood were used. 1·9 represents the correction for the oxygen and nitrogen in physical solution.

If *oxygen content* is being calculated, the correction made to obtain oxygen combined with hæmoglobin is to subtract 1·5 ml. for arterial blood and 1·3 ml. for venous blood. If oxygen only has been measured (see below), the corrections for oxygen in physical solution required to give the oxygen actually combined with hæmoglobin, are 0·5 ml. per 100 ml. for blood saturated at 20° C., 760 mm. pressure, 0·2 ml. for arterial blood and 0·1 ml. for venous.

**Note.** The oxygen can be absorbed by means of an alkaline hydrosulphite solution. Weigh out 10 grams of sodium hydrosulphite and 1 gram of sodium anthraquinone β-sulphonate. Place these in a beaker, add 50 ml. of N potassium hydroxide and stir for a few seconds. Filter quickly through cotton wool. Deaerate in the gas-apparatus and transfer to a bottle and cover with oil. The two solids can be mixed, powdered in a mortar, and kept thus, 11 grams being weighed when required. The solution must be used on the day on which it is prepared, even if it be kept under oil.

For absorption of oxygen, place 2 ml. of the hydrosulphite solution in the cup C and slowly draw in about 0·5 ml., doing this so slowly that a thin film of solution is produced on the wall of the pipette. Run in a little mercury in a fine stream to break any column of solution above the gas. Only 0·02 to 0·03 ml. of nitrogen gas should be left. Run a further 0·5 ml. of hydrosulphite through this bubble. Now draw the hydrosulphite into the side tube F and read the volume of residual gas. This subtracted from the reading of oxygen + nitrogen, gives the oxygen liberated. Correct to N.T.P. as described.

INTERPRETATION

The oxygen content of the arterial blood for males has been estimated as being from 14 to 24 ml. per 100 ml., averaging about 19 ml., the corresponding range for venous blood being 10 to 18 ml., averaging 14 to 15 ml., from which it is seen that the amount of oxygen utilized by the tissues is about 20 per cent. of that present in the arterial blood.

Percentage utilization

$$= \frac{O_2 \text{ content of arterial blood} - O_2 \text{ content of venous blood}}{O_2 \text{ content of arterial blood}} \times 100$$

$$= \frac{19 - 14{\cdot}5}{19} \times 100 = 23 \text{ per cent.}$$

Figures for female blood are lower because of the smaller content of hæmoglobin, being about 10 to 15 per cent. below the values for male blood. The oxygen saturation of arterial blood is only about 5 per cent. short of full saturation, being not less than 94 per cent. saturated, whilst that for venous blood is usually in the range 70 to 75 per cent.

An inadequate supply of oxygen to the tissues is termed *anoxia*, four types of which have been recognized (Barcroft, 1920 ; Peters and Van Slyke, 1932). The more limited term *anoxæmia*, that is, a diminished supply of oxygen to the blood, is also used.

1. **Anoxic anoxia,** in which, although oxygen capacity is normal, saturation of arterial blood is less than normal. This may be due to shortage of oxygen at high altitude ; to the rapid shallow breathing which may occur in some lung diseases such as pneumonia ; as a result of obstruction to the entry of oxygen into the alveoli which may occur in a number of diseases of the respiratory tract, emphysema, lung congestion, bronchitis ; in congenital heart disease, when some blood passes from the right side of the heart to the left thus missing the pulmonary circulation.

2. **Anæmic anoxia,** in which, although oxygen saturation is normal, the arterial blood has a reduced oxygen capacity because some of the hæmoglobin has been rendered non-functioning as a result of its conversion into substances such as carboxyhæmoglobin, methæmoglobin, and sulphæmoglobin ; or because as a result of anæmia there is less hæmoglobin than in normal persons.

3. **Stagnant anoxia,** as a result of some circulatory deficiency. Arterial oxygen capacity may be normal, as may arterial oxygen content, so that saturation of arterial blood is normal, but because of the reduced circulation an increased amount of oxygen is removed by the tissues so that the oxygen content of venous blood and hence the saturation of venous blood is below normal. This is most frequently seen in decompensated heart disease, but may be present in shock.

4. **Histotoxic anoxia** is the term given by Peters and Van Slyke to conditions in which tissue cells are unable to utilize oxygen normally. It may be found in poisoning, for example, by alcohol, and by potassium cyanide.

*Cyanosis* has been shown not to appear until 5 grams of hæmoglobin per 100 ml. of arterial blood is present either as reduced hæmoglobin, or as one of the other blood pigments such as methæmoglobin or sulphæmoglobin.

## VITAL CAPACITY

In each respiration only about 500 ml. of air enters and leaves the body, and of this only about 350 ml. actually enters the lungs to mix with the air already there. This 500 ml. is termed the *tidal air*. However, after a normal expiration a further 1,000 to 1,500 ml. of air can be forced out, the *supplemental air*. There still remains rather more than a litre of air, *residual air*, in the lungs, which cannot

ordinarily be forced out. Furthermore, when, after a normal expiration, as deep an inspiration as possible is taken, $2\frac{1}{2}$ to 3 litres of air can be inhaled. The difference between this and the tidal air is referred to as the *complemental air*.

The *vital capacity* is the sum of the tidal, supplemental, and complemental airs. It is thus the volume of air which can be expelled from the lungs after a maximum inspiration. For its measurement the person fits a mouthpiece, takes as deep a breath as possible, and breathes out through a gas meter (spirometer) which records the volume of gas passing through. The lungs are emptied as completely as possible.

The normal vital capacity averages about 4,000 to 4,500 ml. for men and about 3,000 ml. for women. It naturally varies with size, and has been related to both height and surface area. For men, the normal may be taken as 25 × height in cm., or 2,500 × surface area in square metres ; for women, 20 × height in cm., or 2,000 × surface area in square metres. The type of occupation has a considerable effect of the vital capacity, which is higher in persons leading a more active life. Thus it is high in athletes, lower in sedentary workers.

Measurement of vital capacity has been used mainly in lung diseases, but also in heart disease. In these it is naturally reduced, sometimes very considerably.

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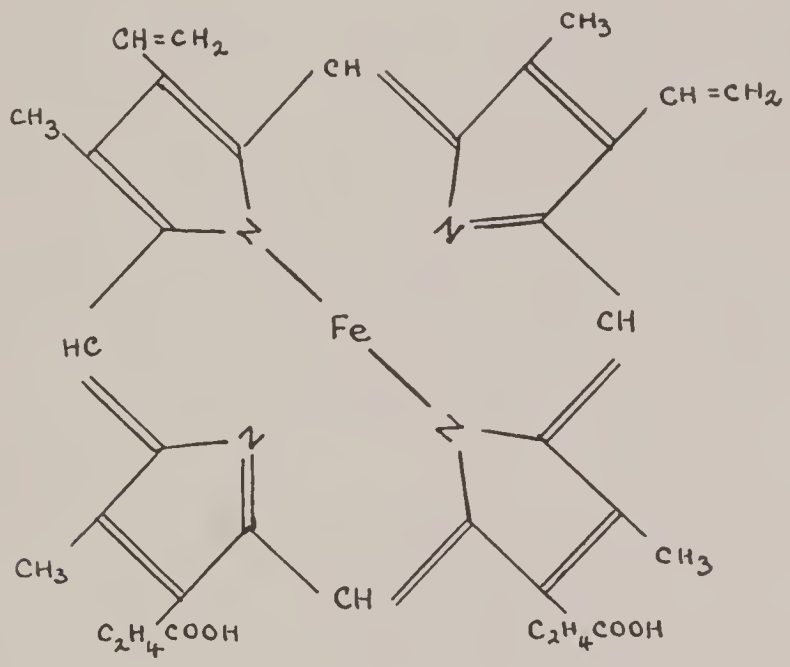
## *CHAPTER XXI*

# HÆMOGLOBIN AND RELATED COMPOUNDS

Hæmoglobin is present to the extent of some 14 to 16 grams per cent. in blood, normally occurring entirely in the red cells. It is a conjugated protein, composed of a protein, globin, and an iron-containing prosthetic group, hæm, the structure of which has been determined and may be represented as shown, the iron being in the ferrous state. Oxidation converts hæm into hæmatin (ferrihæm), which,




Hæm (Ferrihæm)

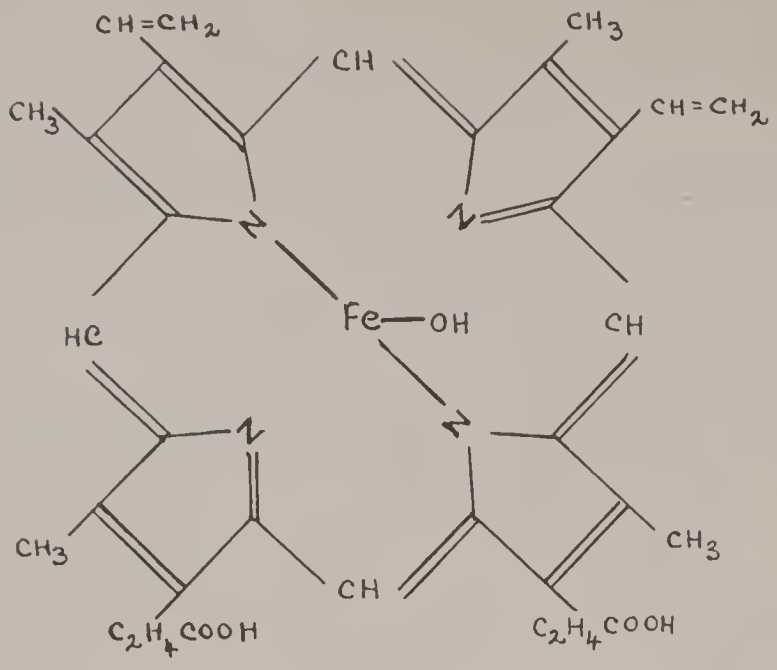
when linked with the protein globin, gives methæmoglobin in which the iron is in the ferric form and which can be prepared directly from hæmoglobin by oxidation. There are accordingly both ferrous and ferric compounds in hæmoglobin and its derivatives.

Hæmoglobin and substances derived from it can be divided into the following groups of compounds representing different stages in their degradation.

1. Hæmoglobin and related iron containing chromoproteins, such as oxyhæmoglobin, methæmoglobin, carboxyhæmoglobin, sulphæmoglobin.

2. When the prosthetic group is removed from the globin we have the group of iron-containing compounds typified by hæm and hæmatin shown above.

3. When the iron is removed from the second group of substances




Hæmatin (Ferrihæm hydroxide)

we obtain the porphyrins, in which the ring of four pyrrole groups is still intact.

4. When the ring is opened we get open chain compounds built up from the four pyrrole groups. These include the bile pigments, bilirubin and related compounds, biliverdin, urobilinogen and so on.

## HÆMOGLOBIN AND RELATED CHROMOPROTEINS

*Hæmoglobin* or *Reduced Hæmoglobin*, as we have seen, is the pigment present in the red blood cells and is a combination of the protein, globin, with the ferrous iron-containing compound, hæm. It would seem that in addition to there being different hæmoglobins for different species, there may be more than one in the same animal.

*Oxyhæmoglobin* is formed when a solution of hæmoglobin is shaken with air. The iron present is not oxidized, but remains in the ferrous state. The union is molecular, the oxygen uniting and dissociating as $O_2$. One gram of hæmoglobin is usually stated to combine with 1·34 ml. of oxygen at N.T.P. but doubts have been expressed as to the correctness of this figure. Two atoms of oxygen are taken up for each atom of iron. Oxyhæmoglobin is readily converted into hæmoglobin, for example, by ammonium sulphide, sodium hydrosulphite, and similarly loses its oxygen when exposed to a low oxygen pressure.

*Carboxyhæmoglobin* is formed by the action of carbon monoxide on hæmoglobin and oxyhæmoglobin. Since carboxyhæmoglobin is more stable than oxyhæmoglobin, carbon monoxide is able to displace oxygen from the latter, so that when air containing small amounts of carbon monoxide is breathed for a period carboxyhæmoglobin tends to accumulate in the blood. However, once the person stops breath-

ing carbon monoxide, carboxyhæmoglobin disappears from the blood fairly rapidly with re-formation of oxyhæmoglobin, a point which should be remembered when investigations are being carried out on suspected carbon monoxide poisoning cases. For the same reason blood should be taken under oil and not shaken in contact with atmospheric oxygen. The colour of carboxyhæmoglobin is somewhat different from that of oxyhæmoglobin, being more pink or carmine. Carboxyhæmoglobin is unaffected by the reducing agents mentioned above which convert oxyhæmoglobin to hæmoglobin in the cold. Heat and a strong reducing agent are necessary to convert carboxyhæmoglobin to hæmoglobin.

The term carbonyl hæmoglobin has been preferred by some workers and is more strictly accurate but carboxyhæmoglobin is in general use.

*Nitric oxide hæmoglobin* can also be formed, and, like carboxyhæmoglobin, has a pinker colour than oxyhæmoglobin, but differs from carboxyhæmoglobin in retaining this pink colour when dilute solutions of it are boiled. Whether it has any significance clinically is doubtful but it has been said to be formed in some cases of pneumonia. It is present in meats cured with nitrites and nitrates and is responsible for the typical colour.

*Methæmoglobin* is formed by oxidation of hæmoglobin and oxyhæmoglobin, for example, by the action of potassium ferricyanide on the latter. The iron is present in the ferric state and methæmoglobin is not able to transport oxygen in the way oxyhæmoglobin can, for although it is converted to hæmoglobin by reducing agents such as ammonium sulphide, sodium hydrosulphite, Stokes' reagent, it does not, like oxyhæmoglobin, give up its oxygen when exposed to low oxygen pressures. Methæmoglobin has a rather characteristic dark brownish colour, giving to urine and to blood which contain it in appreciable amounts a fairly typical colour.

*Sulphæmoglobin* has been shown to contain one more sulphur atom in its molecule than does hæmoglobin, and appears to be an addition product of hæmoglobin. It does not combine reversibly with oxygen and is not reconverted by reducing agents to hæmoglobin, differing in this respect from methæmoglobin.

*Globin-hæmochromogen.* When a dilute solution of blood is warmed with alkali, methæmoglobin is formed and decomposes into globin and hæmatin. The globin is denatured by the alkali, and on adding a reducing agent, for example, sodium hydrosulphite, the hæmatin is reduced to hæm, which combines with the denatured globin forming a globin-hæmochromogen, another chromoprotein.

*Methæmalbumin, Ferrihæmalbumin.* A number of workers had reported the presence of hæmatin in serum in some pathological conditions before Fairley (1941) showed that this was really a pigment formed by the linkage of hæmatin with plasma albumin. This he termed methæmalbumin. Accordingly the iron is in the ferric form. Reduction with ammonium sulphide in concentrated solution (Schumm's test, see p. 399) gives an albumin-hæmochromogen. Serum or plasma containing an appreciable amount of methæm-

albumin has a rather charaeteristic colour, resembling that of bilirubin, but distinguishable by those familiar with both.

*Myohæmoglobin, Myoglobin.* Myohæmoglobin, or musele hæmoglobin, as its name suggests is a pigment—present in muscle—which is similar to hæmoglobin. Its molecular weight, however, is only a quarter of that of hæmoglobin. It forms a series of derivatives similar to oxy, carboxy, and methæmoglobin.

### Occurrence in Cells, Plasma and Urine

Hæmoglobin being an intracorpuseular pigment does not normally oeeur in appreeiable amounts in plasma. However, since it is not easy in praetiee to obtain serum or plasma completely free from oxyhæmoglobin, faint bands ean often be seen on eareful spectroseopie examination. With careless technique gross hæmolysis may oecur. It is important to bear this in mind when examining blood in eases where intravaseular hæmolysis is suspeeted. Conditions in whieh hæmoglobin is present in plasma *in vivo* are referred to later (p. 404). Hæmoglobin can be filtered by the glomeruli when present in plasma if its eoneentration exeeeds a eertain low threshold value. Aeeordingly it is found in urine in eonditions in which there is appreeiable intravaseular hæmolysis.

Methæmoglobin and sulphæmoglobin are not found in normal blood but may be formed intraeorpuseularly from hæmoglobin. So long as the eells are intact they will not appear in the urine. However, if sueh eells hæmolyse intravaseularly, methæmoglobin is liberated and ean be filtered by the glomeruli in the same way as hæmoglobin, and so is found in the urine. Hæmoglobin may also be eonverted into methæmoglobin after it has been passed in the urine. Methæmoglobin may thus be found in eells, plasma, and urine. The presenee of sulphæmoglobin in the urine does not appear to have been reported.

Methæmalbumin, being formed by linkage of hæmatin and plasma albumin, is found in plasma, but is not filtered by the glomeruli and so does not appear in the urine.

## Identification of the Pigments

### Spectroscopic Examination

In examining speeimens for the presenee of these pigments, spectroseopie examination provides valuable evidence, particularly when eombined with ehemieal tests.

Many laboratories will only possess a direet vision speetroseope. In its simplest form this has a movable eyepieee which ean be foeussed and a slit which ean be opened and shut. Some instruments have a seale on which the position of lines ean be noted for comparison purposes, and with some poeket direet vision spectroscopes it is possible to observe two solutions simultaneously. By means of a small prism eovering half the slit and a small mirror at the side, two spectra can be observed one above the other, or a speeimen can be observed at the same time as the spectrum of ordinary light. Alternatively the small prism can be moved so as to get lines of full width

using the whole of the slit to observe the specimen which is being examined.

In using these instruments correct adjustment of the slit and proper focussing are essential if reliable observations are to be made. A narrow slit is usually required. Make the necessary adjustments of slit and eyepiece by observing some rather bright ordinary light. The spectrum should be clear and sharp with the Fraunhöfer lines sharply defined. The liquid under observation may be placed in a test tube, $\frac{3}{4}$ or 1 inch in diameter but it is more convenient to use

TABLE XXVIII

*Absorption Bands of Hæmoglobin and Derivatives*

Wavelengths of Maximum Absorption in Millimicrons

| | $\alpha$ | $\beta$ | $\gamma$ | $\delta$ |
|---|---|---|---|---|
| Hæmoglobin | 555 | 430 | — | — |
| Oxyhæmoglobin | 577 | 541 | 413 | — |
| Carboxyhæmoglobin | 570 | 535 | 418 | — |
| Methæmoglobin | 630 | 500 | 406 | — |
| Alkaline Methæmoglobin | 600 | 577 | 540 | 411 |
| Sulphæmoglobin | 618 | 577 | 541 | — |
| Methæmalbumin | 623–624 | 540 | 501 | — |
| Myohæmoglobin | 582 | 543 | — | — |
| Cyanhæmoglobin | 540 | 414 | — | — |
| Globin hæmochromogen | 558 | — | — | — |
| Albumin hæmochromogen | 558 | 529 | — | — |
| Acid Hæmatin (in glacial acetic acid) | 632 | 540 | 510 | 400 |
| Alkaline hæmatin (in 10 per cent. aqueous NaOH) | 580 | — | — | — |
| Deuteroporphyrin (in 25 per cent. HCl) | 591 | — | 548 | 404 |
| Coproporphyrin (in 25 per cent. HCl) | 593·5 | (574) | 550·5 | 405 |
| Uroporphyrin (in 25 per cent. HCl) | 597 | (577) | 553·5 | 410·5 |
| Zinc uroporphyrin | 577 | 541 | — | — |
| Protoporphyrin (in 25 per cent. HCl) | 602·5 | 582 | 557·2 | 411 |
| Urobilin | 490 | — | — | — |
| Zinc urobilin | 506·5 | — | — | — |

containers with parallel sides of which a series with varying depths of view is particularly valuable. In addition to the proper adjustment of the spectroscope it is important to view through the correct depth for the particular liquid under investigation. With experience this can be gauged with reasonable accuracy, but even then it is often advisable to view through several depths, or to use a series of dilutions. With blood a dilution of 1 in 50 is often used. A useful device is to put some of the blood into a 6 × 1 inch test tube and add water without mixing. A concentration gradient is thus obtained so that by moving the spectroscope slowly up the tube the liquid can be viewed at increasing dilution and the most satisfactory one quickly found for noting the lines present.

Hartridge's reversion spectroscope used by some laboratories is described later.

The bands of the various substances mentioned are given in Table XXVIII, being expressed in millimicrons. The spectra are shown in Fig. 68.

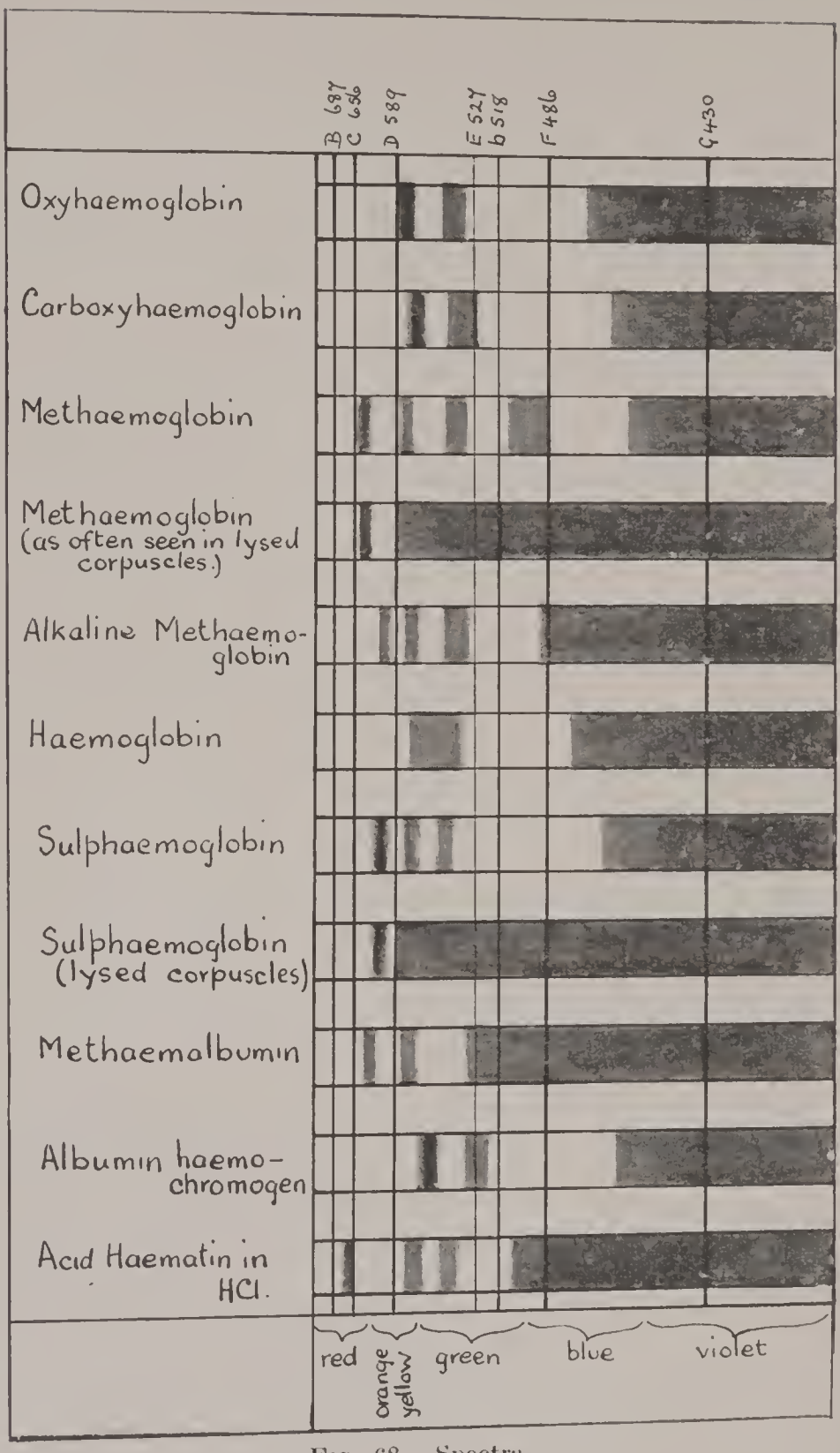



FIG. 68. Spectra.

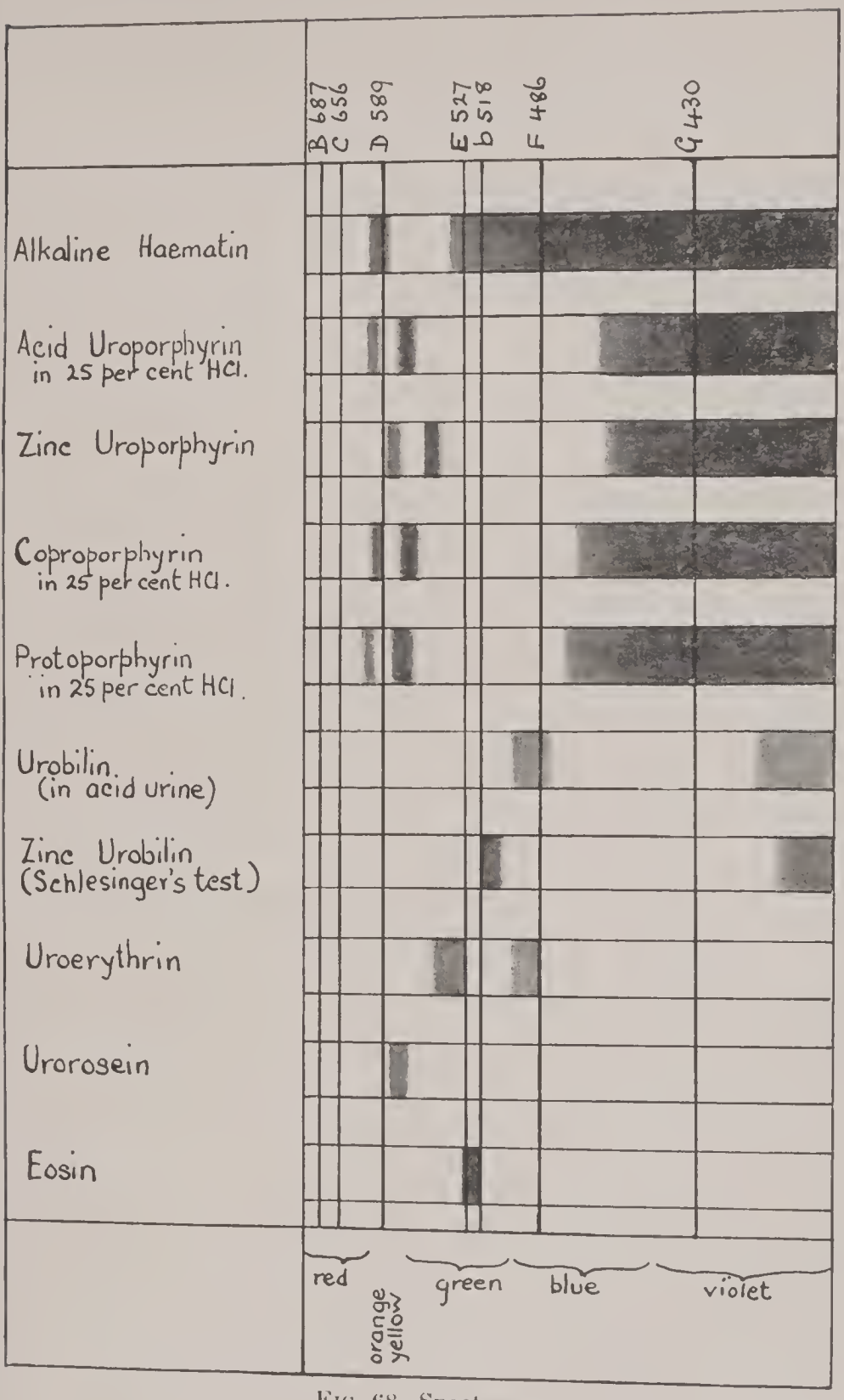



FIG. 68. Spectra.

The two bands in the yellow and the green are particularly characteristic of *oxyhæmoglobin* ; it has a third band in the violet which is not so easily seen. The α band is narrower and more intense than the β and lies very near to the D line.

*Hæmoglobin* shows a broad, not very intense band, centred between the α and β bands of oxyhæmoglobin, covering the whole of the space between them and part of the bands themselves. *Carboxyhæmoglobin* has two bands similar to those of oxyhæmoglobin but displaced slightly towards the violet end of the spectrum. *Methæmoglobin*, *sulphæmoglobin*, and *methæmalbumin*, all show a narrow band of relatively weak intensity in the red. In spectroscopic examination of blood very careful observation should be made for such a band. Its presence may sometimes be overlooked unless blood only moderately diluted is used, or a deep layer of blood diluted 1 in 50 is observed, when only the α band in the red will be seen. It will be noticed that the position of this band in the red is slightly different in the three cases. It is not easy always to see these differences on a direct vision spectroscope but they can be shown in the reversion spectroscope. Differentiation is also possible because of their different behaviour towards reducing agents. The spectra of *globin* and *albumin hæmochromogens* are characterized by two bands in the yellow green, the α band, situated between the two characteristic bands of oxyhæmoglobin, in each case being very much more intense than the β which is rather faint. Their α bands are also considerably more intense than that of oxyhæmoglobin.

*Myohæmoglobin* and its derivatives give spectra similar to those of the corresponding derivatives of hæmoglobin, except that the bands are displaced a little towards the red. Thus the position of the α bands of oxy, carboxy, and metmyohæmoglobin are 581–2, 579, and 636 millimicrons respectively (Bywaters *et al.*, 1941).

Since the pigments in some cases may be either intracorpuscular or extracorpuscular, it may be of interest to study both whole blood and plasma.

**Use of the Hartridge Reversion Spectroscope** (Hartridge, 1914, 1922). In this instrument two spectra of the solution used are formed, with one reversed. By means of an adjustment it is possible to move one of these so that any particular band of one spectrum can be made to touch any band of the other. Normal blood is placed in the instrument and the α bands are made to overlap. Then blood suspected, for example, of containing carboxyhæmoglobin, is introduced. If this is present the bands will no longer coincide since there will have been a slight displacement towards the violet. This can be made more sensitive with special care and filters and the instrument can be used to give a quantitative estimation by calibrating it with diluted blood containing known percentages of saturation with carbon monoxide.

The instrument can be used in the same way for contrasting the bands in the red of methæmoglobin and sulphæmoglobin. By oxidizing a suitable dilution of blood with a crystal of potassium ferricyanide, methæmoglobin is formed, and is placed in the instru-

ment which is then adjusted so that the bands in the red overlap. The blood under test is then substituted. If it eontains methæmoglobin the bands again overlap but do not do so if sulphæmoglobin is present or, using plasma or serum, if methæmalbumin is present. A solution can be prepared by adding to 10 ml. of normal blood diluted 1 in 100, 0·1 ml. of 0·1 per eent. phenylhydrazine in water and 1 drop of water saturated with hydrogen sulphide. This forms sulphæmoglobin. If methæmalbumin is introdueed its α band will overlap neither of these prepared solutions of methæmoglobin or sulphæmoglobin. It may also be noted that when carbon monoxide is passed into a solution of sulphæmoglobin its α band moves towards the violet whereas that of methæmoglobin is not affeeted.

## Action of Reducing Substances

In the great majority of eases it is possible to identify a blood pigment by a eombination of speetroseopie examination using a direct vision speetroseope, with study of the effect of redueing agents. The blood is first observed earefully with the spectroseope using a wide range of dilutions. If no band in the red is seen then methæmoglobin and sulphæmoglobin ean be reported as not deteeted. In the case of methæmalbumin in serum or plasma, reduetion is still done since in some eases in whieh direet examination is negative, the presenee of the albumin hæmoehromogen thus formed, ean be detected spectroseopieally, sinee its α band is so mueh more intense than any of the bands of methæmalbumin.

Of redueing agents the following are used.

*Ammonium sulphide* may either be bought (for example, British Drug Houses Analar) or prepared by saturating 33 per eent. ammonia with hydrogen sulphide and mixing this with one quarter of its volume of 33 per eent. ammonia. This reduees oxyhæmoglobin and methæmoglobin to redueed hæmoglobin, so that the α band in the red in the spectrum of methæmoglobin is removed. Carboxyhæmoglobin and sulphæmoglobin are not affeeted. If the presence of methæmalbumin is being investigated reduetion of plasma or serum is earried out. This is *Schumm's test.* Plaee some serum or plasma in a test tube, cover with ether and add one-tenth its volume of concentrated ammonium sulphide solution. Examine speetroscopieally looking particularly for the α band intermediate between the α and β bands of oxyhæmoglobin. Neither oxyhæmoglobin nor methæmoglobin give a hæmochromogen when treated in this way.

*Sodium hydrosulphite* (sodium dithionite) $Na_2S_2O_4$ added in the solid form acts similarly.

*Stokes' reagent*, ammonium ferrotartrate. Weigh out 2 grams of ferrous sulphate and 3 grams of tartarie acid. Dissolve eaeh in water, mix, and make to 100 ml. with water. For use take a small amount and add ammonia, S.G. 0·88, drop by drop until the precipitate whieh first forms is redissolved. This acts on oxyhæmoglobin and methæmoglobin but not on methæmalbumin.

### Action of Alkalies

*Ammonia.* One to 2 drops added to 3 to 5 ml. of the diluted blood converts methæmoglobin to alkaline methæmoglobin, which has no band in the red (see Table XXVIII, Fig. 68). Sulphæmoglobin is not affected by such treatment.

Oxyhæmoglobin and carboxyhæmoglobin also behave differently towards alkali. Mix equal volumes of blood and 25 per cent. sodium hydroxide. Oxyhæmoglobin turns brown whereas carboxyhæmoglobin is unaffected.

## Determination of Hæmoglobin

The measurement of the amount of hæmoglobin in blood is most often carried out in the hæmatology departments of hospital laboratories, so that it will only be considered briefly. For further information, see, for example, Dacie (1950) and Macfarlane (1951). Methods using the photoelectric colorimeter are most frequently used.

### I. Oxyhæmoglobin Method

Wash 0·02 ml. of blood into 4 ml. of 0·04 per cent. ammonia (0·4 ml. of ammonia, S.G. 0·88, made up to 100 ml. with water). Read in the photoelectric colorimeter using a yellow-green filter or transmission at 540 millimicrons. Read the amount of hæmoglobin present on a standard curve prepared as follows :

Take 10 ml. of venous blood from a young, healthy person and deliver into a tube containing 20 mg. of Paul and Heller's anticoagulant (see p. 175) derived from 1 ml. of solution. Add a knife-point of saponin to hæmolyse the cells. Remove the cell envelopes by centrifuging and make a 1 in 100 dilution of the supernatant fluid with 0·04 per cent. ammonia (v/v). Take a portion and saturate with pure carbon monoxide. Seal in a test tube over carbon monoxide and send to the National Physical Laboratory for standardization. Do this at least once a year.

From some of the remainder prepare a 1 in 200 dilution and read in the colorimeter as for the test. Prepare also a 1 in 200 dilution saturated with carbon monoxide and read in the same way. Use the original sample also to prepare dilutions so as to obtain a standard curve. Then from the information received from the N.P.L. it will be possible to fix the hæmoglobin value for a point on this curve. Keep the standard under carbon monoxide at 4° C. in the dark so that it can be used at intervals to check the calibration curve used. In particular this must be done when any change is made in the colorimeter such as replacing a bulb.

The hæmoglobin content of blood can also be determined by estimating the iron content of the blood. See, for example, the titanous chloride method (King, 1951).

### II. Alkaline Hæmatin Method (Clegg and King, 1942)

Add 0·05 ml. of blood to 4·95 ml. of N/10 sodium hydroxide, heat for five minutes in boiling water, and read in the photoelectric

colorimeter using a yellow-green filter or transmission at 540 millimicrons. As standard pure hæmin can be used. Dissolve 37·5 mg. in 1 litre of N/10 sodium hydroxide. This gives a colour similar to that given by blood containing 7·4 grams of hæmoglobin per 100 ml. On the Haldane scale, 14·8 grams equals 100 per cent., so this standard is 50 per cent. of the average normal.

Gibson and Harrison (1945) have used an artificial standard which has a colour equal to that given by 16·0 grams hæmoglobin per 100 ml. To prepare this dissolve 11·61 grams of chromium potassium sulphate, $CrK(SO_4)_2$, $12H_2O$, showing no signs of efflorescence, 13·1 grams of anhydrous cobaltous sulphate, and 0·69 gram of potassium dichromate in about 500 ml. of distilled water, add 1·8 ml. of N sulphuric acid, boil for one minute, cool, and make up to 1 litre. Dry, anhydrous, cobaltous sulphate must be used. Heat about 30 grams of $CoSO_4$, $7H_2O$ for about two hours in a small porcelain dish at 96° C., breaking up the coarser particles frequently. Then heat overnight at about 400° C. in an electric furnace, transfer hot to a stoppered bottle, cool, and weigh 13·10 grams immediately.

**Normal Range.** The following figures for the normal range are given by Dacie (*Practical Hæmatology*, J. & A. Churchill Ltd.), 1950 :

| | |
|---|---|
| Men | 13·5 to 18·0 grams per 100 ml. |
| Women | 11·5 to 16·4 .. .. .. |
| Infants, at birth | 14·0 to 19·0 .. .. .. |
| Children, one year | 11·2 grams per 100 ml. (average) |
| ten years | 12·9 .. .. .. .. |

## Determination of the Carboxyhæmoglobin present in Blood

Several methods have been used for determining the amount of carboxyhæmoglobin present in blood. They vary widely in sensitivity. One of the simplest and most sensitive is the method of Salt using the photoelectric colorimeter.

### I. Microphotometric Method of Salt (1951)

The blood is diluted with a very weak ammonia solution and read in the photoelectric colorimeter, using a suitable filter, before and after shaking with a little sodium dithionite, $Na_2S_2O_4$ (sodium hydrosulphite). This reduces oxyhæmoglobin to hæmoglobin which gives a larger reading on the colorimeter, whereas carboxyhæmoglobin is unaffected. So the more carboxyhæmoglobin present the smaller the change in reading. The blood is fully oxygenated and the same readings made. By comparing the effect of the dithionite in the two cases a measure of the amount of carboxyhæmoglobin present is obtained.

**Reagents.** 1. Ammonia, 0·007 N (0·1 per cent. v/v ammonia S.G. 0·88).

2. Sodium dithionite, powdered.

**Technique.** Dilute 0·1 ml. of blood to 12 ml. with 0·007 N ammonia. Take twice this volume of blood if the hæmoglobin is between 6 and

12 grams per 100 ml. Divide into two portions of 6 ml. Place one in the colorimeter and read with an orange filter having peak transmission at 600 millimicrons (Ilford No. 607). Add a few granules of solid sodium dithionite, avoiding an excess, and read again. To confirm that complete reduction has occurred, add a further small amount of sodium dithionite and read once more. No further alteration in reading should be noticed. Next bubble oxygen through the other 6 ml. of diluted blood at room temperature for thirty minutes at the rate of six bubbles per second through a glass jet 1 mm. in diameter. Transfer to the colorimeter cell and read. Reduce with sodium dithionite as above and read again.

The percentage of the pigment present as oxyhæmoglobin is given by

$$\frac{E_{\text{Diluted blood reduced}} - E_{\text{Diluted blood}}}{E_{\text{Fully oxygenated diluted blood reduced}} - E_{\text{Fully oxygenated diluted blood}}} \times 100$$

where E stands for extinction coefficient.

If this percentage is subtracted from 100 we obtain the percentage of the pigment present as carboxyhæmoglobin.

The absolute amount of carboxyhæmoglobin can be obtained if a standard curve is available, giving the extinction coefficients for known amounts of hæmoglobin reduced by dithionite.

It is claimed that this method will detect as little as 4 per cent. of the hæmoglobin present as carboxyhæmoglobin.

### II. Tannic Acid Method (Sayers and Yant 1926)

**Qualitative Tannic Acid Test.** Dilute the blood under test and some normal blood, 1 in 5 with water, and add to each a few drops of a 3 per cent. tannic acid solution. Shake well. Normal blood gives a precipitate which is dirty brown, carboxyhæmoglobin a pink one. So the greater the proportion of carboxyhæmoglobin the more pink is the deposit obtained.

#### Quantitative Tannic Acid Method

**Reagent.** Pyrogallol-tannic acid solution. Dissolve 2 grams of pyrogallol and 2 grams of tannic acid in water and make to 100 ml.

**Technique.** Collect blood from an arm into an oxalate tube under paraffin or directly into a 0·2 ml. pipette from a finger if desired. Dilute 1 in 10 with distilled water, and to 1 ml. of diluted blood add 1 ml. of freshly prepared pyrogallol-tannic acid reagent, in a tube similar to those used for preparing the standards. Mix. Stand fifteen minutes and compare with standards.

To prepare standards dilute normal blood 1 in 10 with water and shake to hæmolyse the cells. Saturate about half with carbon monoxide by rotating with coal gas in a separating funnel for about a quarter of an hour. By mixing this with untreated blood, put up a set of tubes containing blood with 0, 10, 20, 30 . . . 90, 100 per cent. saturation of carbon monoxide. Add 1 ml. of pyrogallol-tannic acid reagent to 1 ml. of each. Mix, cover with melted wax and seal off the tubes.

### III. Use of Dilution in Testing for and Determining Carboxyhæmoglobin

On simple dilution, blood containing an appreciable proportion of carboxyhæmoglobin has a distinctly different colour from normal blood similarly diluted. Use a dilution of 1 in 200 in 0·4 per cent. ammonia (v/v with ammonia, S.G. 0·88). Carboxyhæmoglobin has a pink to carmine tinge, oxyhæmoglobin a more yellowish shade of red. By comparing a suspected blood with normal blood and normal blood saturated with carbon monoxide all similarly diluted, quite a sensitive indication is obtained as to whether carboxyhæmoglobin is present in appreciable amounts. The test can be made roughly quantitative by preparing a set of standards of known percentages of saturation with carbon monoxide. On the Lovibond tintometer a disc is obtainable for such diluted blood, ranging from 0 to 100 per cent. saturation. The 0 or normal blood corresponds to a normal blood containing 14·8 grams hæmoglobin on the Haldane scale. So by comparing the blood under test with the disc, and by measuring the hæmoglobin present, which can be done by saturating with carbon monoxide, it is possible to calculate the amount of carboxyhæmoglobin present in the blood.

### IV. Gasometric Method using the Van Slyke Apparatus (p. 354).

The gases are liberated from the blood, and the oxygen and carbon dioxide absorbed in alkaline pyrogallol, leaving carbon monoxide and nitrogen. The carbon monoxide can be absorbed in ammoniacal cuprous chloride solution or a correction made for the nitrogen present.

**Reagents.** 1. Special carbon monoxide reagent. Dissolve 3 grams of saponin and 8 grams of potassium ferricyanide in water, add 4 ml. of lactic acid, and then 3 ml. of caprylic alcohol, and make up to a litre with water.

2. Alkaline pyrogallol solution. Dissolve 10 grams of pyrogallol in 200 ml. of a solution of potassium hydroxide prepared by dissolving 160 grams in 130 ml. of water.

3. Winkler's reagent. Dissolve 40 grams of cuprous chloride and 50 grams of ammonium chloride in water and make up to 150 ml. Before use, mix 3 volumes of this with 1 volume of ammonia, S.G. 0·88.

**Technique.** Collect a specimen of oxalated blood under oil. Run about 8 ml. of the special carbon monoxide reagent into the cup C of the Van Slyke apparatus and deaerate as described previously (p. 387). Bring back into the cup, run 2 ml. of blood, well mixed so as to redistribute the red cells evenly, into the pipette of the apparatus, followed by 6 ml. of the reagent, and liberate the gases, shaking until a constant volume is reached. Place a little mineral oil in cup C and run 1 ml. of pyrogallol solution in underneath it. Then run this into the pipette following with a little mercury in a fine stream to break any column which forms. Carbon dioxide and oxygen are then absorbed, proceeding until a constant volume is obtained. Read this and note the temperature and barometric pressure. Carbon monoxide content can be calculated from this data or the carbon monoxide can be absorbed as follows. Run the liquid in

the pipette into the tube F. Wash cup C with a little distilled water, and from it draw 0·5 ml. of Winkler's reagent into the pipette, and absorb the carbon monoxide. This occurs quickly, after which the volume of remaining gas should be read immediately. The difference between this and the previous reading gives the volume of carbon monoxide present in the blood used.

If necessary, use a greater volume of blood, increasing the volume of the reagents used in the same proportion.

**Calculation.** The corrected volume of gas is obtained as follows.

$$\text{Corrected volume} = \frac{(V_1 - V_2)\,(B - w)}{760\,(1 + 0{\cdot}00367t)}$$

where $V_1$ = observed volume of gas ($CO + N_2$)
$V_2$ = observed volume of gas after absorption of CO. ($N_2$).
B, $w$, $t$, are as before (p. 387).

If absorption of carbon monoxide is not done, the volume of carbon monoxide in ml. per 100 ml. is given by

$$\text{Corrected volume} \times \frac{100}{2} - 1{\cdot}36.$$

HÆMOGLOBIN AND RELATED CHROMOPROTEINS. INTERPRETATION

*Hæmoglobin* is normally confined to the corpuscles but may be present free in the plasma—*hæmoglobinæmia*—when there is appreciable intravascular hæmolysis. This occurs in the severe hæmolytic anæmias, for example, malaria and blackwater fever ; in septicæmia due to hæmolytic streptococcus ; as a result of transfusion with incompatible blood ; in the paroxysmal hæmoglobinurias, whether due to cold, severe exercise (march hæmoglobinuria), or nocturnal hæmoglobinuria ; and from the action of chemicals such as the sulphonamides, phenylhydrazine, arsenic drugs.

It would seem that hæmoglobin appears in the urine when the plasma level is over about 130 mg. per 100 ml.

Hæmoglobin liberated in the plasma is partly excreted as hæmoglobin in the urine, is partly converted into methæmalbumin with hæmatin as intermediary and subsequently to bilirubin.

Fairley (1941) has classified the hæmolytic anæmias into three groups. 1. Those with hæmoglobinæmia and hæmoglobinuria, methæmalbuminæmia, and hyperbilirubinæmia, for example malaria and other severe hæmolytic anæmias given above. 2. Those with methæmalbuminæmia and hyperbilirubinæmia, for example, pernicious anæmia. 3. Those with hyperbilirubinæmia only, for example, acholuric jaundice, in which the hæmolysis is not intravascular.

For hæmoglobin in the urine see also p. 85.

*Methæmoglobin* is most commonly formed from hæmoglobin inside the corpuscles but may be present in the plasma if hæmolysis then occurs. It is found in the urine only if present in the plasma and not so long as it remains in the cells. It may be produced by the action of a number of chemicals, particularly some benzene derivatives, aniline dyes, and drugs. Examples are nitrobenzene,

aniline, acetanilide, antipyrene, phenacetin, sulphonal, and some sulphonamides. Nitrites, chlorates, and permanganates may act similarly. Some cases of methæmoglobinæmia have been thought to be due to absorption of toxins or of nitrites from the action of nitrite-producing bacteria in the intestine, the so-called enterogenous cyanosis. A congenital methæmoglobinæmia has been reported resulting in cyanosis, but is very rare. Methæmoglobin may be formed from hæmoglobin in the acute hæmolytic anæmias mentioned above and lead to a methæmoglobinuria.

An amount of methæmoglobin above 5 grams per 100 ml. of blood results in the presence of a cyanosis visible clinically.

*Sulphæmoglobin* is much less commonly seen than methæmoglobin in the blood. It arises similarly by the action of drugs and aniline derivatives. For example, sulphæmoglobin has been found intracorpuscularly in patients taking sulphanilamide. Sulphæmoglobin is usually intracorpuscular but is said to be liberated into the plasma on occasion, under which circumstance sulphæmoglobinuria should occur, but this does not appear to have been reported.

*Carboxyhæmoglobin* is only of importance in suspected carbon monoxide poisoning. Since hæmoglobin has a much greater affinity for carbon monoxide than for oxygen (about 200 times greater) if air is breathed containing carbon monoxide in one two-hundredth the concentration of oxygen, equal amounts of carboxyhæmoglobin and oxyhæmoglobin will be formed, that is, a concentration of about 0·1 per cent. of carbon monoxide in the air breathed will lead to 50 per cent. of the hæmoglobin being present as carboxyhæmoglobin and will produce marked symptoms within about an hour. With 0·2 per cent. death may occur within a few hours.

In a person with a normal blood count, symptoms may begin to appear when there is about 20 per cent. saturation of the hæmoglobin with carbon monoxide. There may be a feeling of lassitude with some headache. Above this point, symptoms steadily worsen, the headaches become severer, with increasing feeling of fatigue, and muscular weakness, giddiness, fainting, and shortness of breath which are very marked at 50 per cent. saturation. Unconsciousness supervenes between 50 and 70 per cent., with death following soon if exposure to the gas continues. Death follows quickly when 80 per cent. saturation is reached.

*Methæmalbumin.* The presence of this pigment in serum shows that intravascular hæmolysis has occurred (Fairley, 1941). As we have seen, when hæmoglobin is liberated into the plasma, hæmatin is formed during the course of its conversion to bilirubin, and links with plasma albumin to form methæmalbumin. On the other hand, methæmalbumin does not appear to be formed when there is increased breakdown of hæmoglobin in the reticulo-endothelial system. Hence methæmalbumin is found in the plasma in malaria and in many of the acute hæmolytic anæmias in which the hæmolysis is intravascular, and in pernicious anæmia, but not in such conditions as acholuric jaundice in which the hæmolysis is non-vascular.

*Myohæmoglobin* is found in the urine in the crush syndrome. In

accidents when muscles are crushed this pigment is liberated and is excreted in the urine. This may be mistaken for hæmoglobinuria. Red blood cells are not present but the urine deposit contains casts which are stained with this brown pigment. Whether it is responsible for the tubular damage and blockage which is present is not established. It is not surprising, in view of the relatively small molecular weight, that myohæmoglobin is not found in the plasma. It is filtered too quickly by the glomeruli.

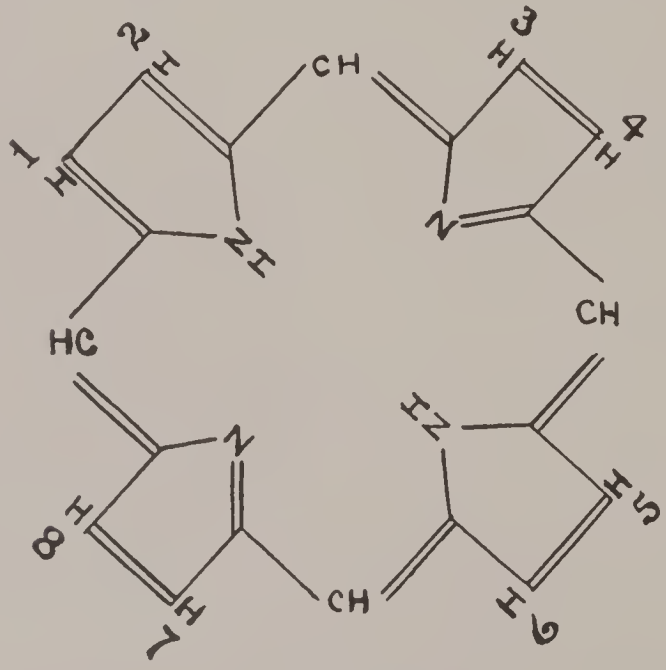
## HÆMATIN

Hæmatin is hæm which has been oxidized and so contains iron in the ferric form. It is a dark powder which does not dissolve in water, but is soluble in alkalies to form alkaline hæmatin and in acids to give acid hæmatin. If diluted blood is acidified with hydrochloric acid or glacial acetic acid and the mixture extracted with ether, an ethereal extract of acid hæmatin is obtained The spectra of acid and alkaline hæmatin are given in Fig. 68. The dark colour of blood found in the stomach in ulcer cases is due to the formation of acid hæmatin.

There were a number of reports that hæmatin was present in the serum in such conditions as malaria, and pernicious anæmia, in which it is now recognized that methæmalbumin is present. It seems probable that hæmatin does not occur free in plasma or serum.

## PORPHYRINS

When iron is removed from the hæmatin type of compound we come to the group of substances known as the porphyrins. These




Porphin

are derivatives of porphin, built up of 4 pyrrole rings linked by methene (= CH —) groups as shown. The positions of single and double linkages in the formula are misleading since the whole molecule is a resonating one and all four pyrroles are equal. The various porphyrins differ as regards the groups substituted for hydrogens in the $\beta$-positions of the pyrrole nuclei, that is in the positions numbered 1 to 8 in the formula given. The most important porphyrin is protoporphyrin, which in the form of its iron complex linked with the protein globin gives hæmoglobin, and with other proteins forms myoglobin, catalase, cytochrome and other respiratory pigments. This protoporphyrin is 1, 3, 5, 8 tetramethyl, 2, 4 divinyl, 6, 7 propionic acid porphin as will be seen on inspecting the formulæ for hæm and hæmatin on pp. 391–2. There are fifteen possible isomers from substituting the eight groups present in protoporphyrin in the $\beta\beta^1$ positions of the pyrrole nuclei of porphin. The majority were synthesized by Hans Fiseher and his collaborators. That present in hæmoglobin and its derivatives was found to be protoporphyrin 9 on Fiseher's list.

If 4 methyl and 4 ethyl groups are substituted there are four possible isomers. These are known as ætioporphyrins of series I, II, III, and IV, of which only compounds of series I and III are found in nature. In series I, the ætioporphyrin is 1, 3, 5, 7 methyl 2, 4, 6, 8 ethyl porphin ; in series III, 1, 3, 5, 8 methyl, 2, 4, 6, 7 ethyl porphin, so that the protoporphyrin of hæmoglobin belongs to series III. Other important members are :

coproporphyrins . 4 methyl groups and 4 propionic acid groups.
uroporphyrins . 4 acetic acid groups and 4 propionic acid groups.

The substance known as hæmatoporphyrin, which can be obtained by adding 2 drops of blood to a few ml. of concentrated sulphuric acid and mixing well, contains 4 methyl groups, 2 propionic acid groups and 2 hydroxyethyl groups ($CHOH.CH_3$). It does not occur in nature except combined in cytochrome C, so that the use of the term hæmatoporphyrinuria is incorrect and the term porphyrinuria or porphyria should be used. Examples of several groups of porphyrin are summarized :

| | | | | |
|---|---|---|---|---|
| Ætioporphryin . | $C_{32}H_{38}N_4$ | $4\ CH_3$ | $4\ C_2H_5$ | |
| Mesoporphyrin . | $C_{34}H_{38}O_4N_4$ | $4\ CH_3$ | $2\ C_2H_5$ | $2\ CH_2CH_2COOH$. |
| Protoporphyrin. | $C_{34}H_{34}O_4N_4$ | $4\ CH_3$ | $2\ CH{=}CH_2$ | $2\ CH_2CH_2COOH$. |
| Deuteroporphyrin | $C_{30}H_{30}O_4N_4$ | $4\ CH_3$ | $2\ H$ | $2\ CH_2CH_2COOH$. |
| Coproporphyrin | $C_{36}H_{38}O_4N_8$ | $4\ CH_3$ | | $4\ CH_2CH_2COOH$. |
| Uroporphyrin . | $C_{40}H_{38}O_4N_{16}$ | $4\ CH_2COOH$ | | $4\ CH_2CH_2COOH$. |
| Hæmatoporphyrin | $C_{34}H_{38}O_4N_6$ | $4\ CH_3$ | $2\ CH(OH)CH_3$ | $2\ CH_2CH_2COOH$. |

Coproporphyrins and uroporphyrins of series I and III are known physiologically. Coproporphyrins I and III have the four methyl groups, uroporphyrins I and III the four acetic acid groups, in the same positions as the four methyl groups of ætioporphyrins I and III respectively. Hæmoglobin and related substances are formed from

protoporphyrin 9 which belongs to the type III series. Compounds of types I and III cannot be converted into each other without opening up the ring and resynthesis. It is held at present that coproporphyrin III is formed in the course of hæmoglobin synthesis. A small amount of coproporphyrin I is possibly formed as a side product. This, probably together with small amounts of coproporphyrin III which have not been utilized, passes into the blood and is excreted by the liver in the bile and by the kidneys in the urine. Up to 120 $\mu$g. has been given as the amount excreted daily in the urine (Lemberg and Legge, 1949) and consists of a mixture of coproporphyrins I and III (20 to 40 per cent. of type III) with a small amount of uroporphyrin. Recently, however, evidence has been adduced to show that some porphyrins are present as colourless non-fluorescing chromogens so that when these are converted into porphyrins a normal daily excretion of 50 to 250 $\mu$g. (mean 175) is found (Watson, 1951). In the fæces 150 to 400 $\mu$g. are excreted daily (Lemberg and Legge). This is derived in part from the action of bacteria on hæmatin compounds present in foods. Coproporphyrin I forms the greater part of the faecal porphyrins. Uroporphyrin is not found in normal fæces. Coproporphyrin and traces of protoporphyrin are excreted in the fæces even on a vegetarian diet. The amount excreted, however, is augmented by ingestion of meat or blood. An earlier view that deuteroporphyrin only appears in fæces after ingestion of blood or following gastro-intestinal hæmorrhage has been disproved, as it is also excreted in the bile, and cannot therefore be employed to detect intestinal bleeding.

### Increase in Porphyrin Excretion

The conditions in which an increased excretion of porphyrins is found can be divided into two groups, termed : (1) porphyrinuria. (2) the porphyrias.

**Porphyrinuria.** An increase in urinary porphyrin is found in several diseases and as a result of the action of some drugs and poisons. There is thus a small increase which rarely exceeds 1 mg. daily in liver diseases (mainly coproporphyrin III), in hæmolytic anæmias and polycythæmia, and in fevers (mainly coproporphyrin I).

Determination of urine porphyrins is useful in lead poisoning (Kench, Gillam and Lane, 1942) and as an additional control of hazard in industry. The amount excreted in lead poisoning may rise to 5 to 10 mg. a day and appears to be a rough guide to the severity of the condition. It was formerly thought that only coproporphyrin III was excreted in lead poisoning, but Kench, Lane, and Varley (1952) have shown that an increased amount of coproporphyrin I is also present.

**Porphyrias.** These are diseases in which the excretion of uroporphyrin or its precursors is greatly increased. There are two types, congenital and acute idiopathic.

(*a*) *Congenital porphyria*, which is inherited as a Mendelian recessive condition, presents soon after birth and is characterized by the formation and excretion of large quantities of uroporphyrin and

coproporphyrin, both mainly type I, deposition of uroporphyrin in the bones and light sensitization of the skin. It is a chronic disease and its victims often live to late middle age. It is often associated with increased hæmolysis of red cells. The total amount of porphyrins excreted is most often in the region 50 to 100 mg. daily but there have been cases with much higher figures.

(*b*) *Acute idiopathic porphyria* presents as recurrent nervous, neuromuscular and abdominal attacks and is inherited as a Mendelian dominant. The disease usually begins in the third decade of life and is rapidly fatal after one or two attacks in most cases, though it can be milder and more chronic. The condition is sometimes precipitated by certain drugs, for example, sulphonal. In early attacks the abdominal signs are often misdiagnosed as abdominal emergency and examination of the urine for porphyrins is valuable in any case which presents with other nervous manifestations. The daily porphyrin excretion is most often from 10 to 30 mg. daily and consists mainly of uroporphyrin III (of Waldenström, 1935). The presence of porphobilinogen (see p. 410) is diagnostic of this condition.

### Tests for Porphyrins in Urine and Fæces

**Spectroscopic Examination.** In porphyrinuria the urine does not appear red in colour and does not show any absorption spectra. On the other hand, in the porphyrias it is usually dark reddish-brown. Examination with the direct vision spectroscope in acute idiopathic porphyria shows the zinc-uroporphyrin absorption spectrum with bands at 577 and 541 millimicrons. This may be confused with the bands of oxyhæmoglobin, but acidification with hydrochloric acid to 10 per cent. (w/v) splits the zinc complex, and develops the characteristic acid porphyrin spectrum with bands at 597 and 553 millimicrons.

**Ultra-violet Fluorescence.** The porphyrins excreted in congenital porphyria are usually free so that examination of untreated urine shows a red fluorescence in ultra-violet light. This does not apply to acute idiopathic porphyria in which the urine contains porphobilin and will no longer fluoresce. In porphyrinuria the amount excreted is so small that fluorescence cannot be seen unless the porphyrin is extracted into ether and so concentrated. A suitable source of ultra-violet light is a 100 watt Mercra lamp (B.T.H.).

**Extraction of Porphyrins.** Ether-soluble porphyrins, which include all except uroporphyrins, can be extracted directly from urine with ether after acidification, or from fæces, bile, or serum, with mixtures of ether and acetic acid. Collect twenty-four-hour specimens of urine, preferably for three to four days since there are wide variations in the amount of porphyrins excreted. To about 300 ml. of urine add one-tenth its volume of glacial acetic acid and then approximately 100 ml. of ether. Shake in a stoppered Pyrex separating funnel for two to three minutes. Withdraw the lower layer and again extract with 100 ml. of ether. Pool the extracts, wash three times with distilled water and extract the porphyrin repeatedly with 5 per cent. w/v hydrochloric acid, that is until no more red fluorescence can be

seen in the ether layer. Use about 20 ml. of the acid for the first extraction, then follow with 10 ml. portions. Neutralize the acid extract to Congo red with saturated sodium acetate solution, and again extract the porphyrins twice with 100 ml. of ether. Wash the ether extract with water, and extract the coproporphyrin into 0·25 per cent. w/v hydrochloric acid in the same way and determine fluorimetrically. For details of the construction of a simple fluorescence comparator see Rimington (1943).

The following points must be borne in mind. Use Pyrex glassware, glass distilled water, and peroxide-free ether throughout. Keep all porphyrin solutions out of the light. If any precipitate forms at the interface, redissolve this in acetic acid and carry through the whole extraction on it. Scrutinize all extractions and washings under ultra-violet light to check that washings are porphyrin free. It is essential in the analysis of fæces and bile to transfer between acid and ether in order to remove bile pigments.

After removal of ether-soluble porphyrins, uroporphyrins remain, and can be concentrated by adsorption on to calcium phosphate, and subsequent elution with normal hydrochloric acid. For details of the technique see Sveinsson, Rimington and Barnes, 1949.

## Porphobilinogen

In addition to uroporphyrin, the urine of acute idiopathic porphyria also contains a dipyrrolic substance, porphobilinogen, a positive test for which is pathognomic of the disease. This test may be carried out as follows (Watson and Schwartz, 1941).

Mix equal volumes of urine and Ehrlich's reagent (0·7 gram of *p*-dimethylaminobenzaldehyde, 150 ml. concentrated hydrochloric acid, and 100 ml. distilled water). Allow to stand for three minutes. To this add saturated aqueous sodium acetate solution (2 volumes) and leave three minutes. Add a few ml. of chloroform and shake thoroughly. Porphobilinogen forms a red aldehyde compound, which unlike that formed by urobilinogen, is insoluble in chloroform. So any red colour remaining in the aqueous phase after a second extraction with chloroform, constitutes a positive test for porphobilinogen and is indicative of acute idiopathic porphyria.

**Notes.** The sodium acetate must be saturated to bring the urobilinogen completely into the chloroform. Furthermore, stale urines must first be reduced with ferrous sulphate and sodium hydroxide under liquid paraffin, as described under urobilinogen (p. 235).

## Chromatography of Porphyrins

Recently separation of porphyrins has been achieved by use of lutidine in single strip chromatograms. See Nicholas and Rimington (1949, 1951); Nicholas (1951). Chu *et al.* (1951) have also succeeded in separating many of the porphyrin methyl esters. This latter method involves preliminary methylation.

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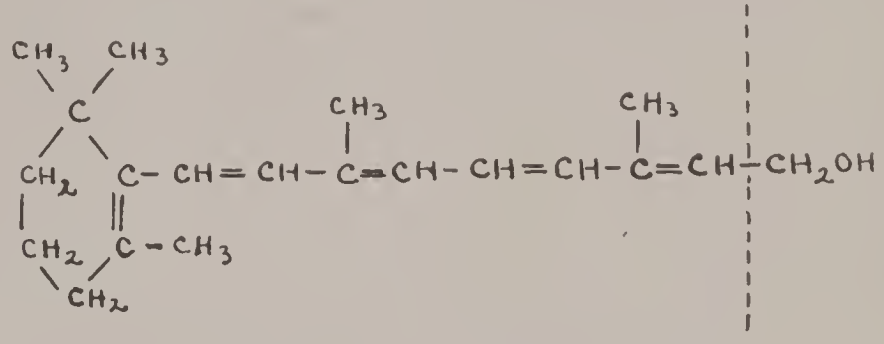
*CHAPTER XXII*

# VITAMINS

Laboratory methods for investigating vitamin deficiencies have used the determination of the vitamin in blood and urine, either under ordinary conditions or after test doses ; the determination in blood and urine of metabolic products derived from the vitamin, or of some substance in blood or urine the concentration of which is influenced by the amount of the vitamin which has been taken in the food. Examples of all these will be given. For a comprehensive survey of chemical methods for studying vitamin deficiencies, see Jones (1951).

## VITAMIN A AND CAROTENES

Vitamin A is obtained from foods either as the vitamin itself or from the plant pigments, carotenes, which can be converted into vitamin A by the liver. Carotenes which can be used in this way are $\alpha$, $\beta$, $\gamma$, carotenes and cryptoxanthine. The formula for vitamin A is



Vitamin A

If we write this as $X_1 - CH_2OH$ where $X_1$ represents the whole of the formula to the left of the dotted line, then the carotenes can be represented by the formula $X_1 - CH = CH - X_2$. In the case of $\beta$-carotene $X_2$ is the same as $X_1$ so that the formula is symmetrical. In the other three carotenes this is not so, though the differences in $X_2$ are small and do not occur in the chain but in the ring. Theoretically it should be possible to obtain two molecules of vitamin A from one molecule of $\beta$-carotene but not from the other carotenes. However, it appears that only one molecule of vitamin A is obtained from one molecule of each of the carotenes.

It is thus evident that both vitamin A and carotenes are important in ensuring an adequate supply of vitamin A to the tissues though in the case of the carotenes this is true only so long as the liver is able to convert them into vitamin A.

**Determination of Vitamin A and Carotenes in Serum using the Carr Price Reaction** (Kimble, 1938-39 ; Kaser and Stekol, 1943)

Proteins are precipitated with alcohol and the vitamin A and carotenes extracted into petroleum ether. The intensity of the yellow colour due to the carotenes is read directly in the colorimeter. The petroleum ether is evaporated off, and the residue taken up in chloroform. Carr Price reagent is added and the amount of blue colour produced is read. Since carotenes also give some colour, a correction for this has to be made in order to obtain the amount of colour due to the vitamin A present.

**Reagents.** 1. Absolute alcohol.
2. Petroleum ether, b.p. 40° to 60° C.
3. A cylinder of carbon dioxide.
4. Chloroform.
5. Acetic anhydride. Use good quality analytical reagent.
6. Carr Price reagent. This is a 25 per cent. solution (w/v) of antimony trichloride in chloroform. Keep at room temperature in a tightly-stoppered brown bottle, filtering before use if necessary.

**Technique.** Pipette 3 ml. of serum or plasma into a stoppered 25 ml. measuring cylinder and add 3 ml. of absolute alcohol, slowly drop by drop with shaking, in order to obtain a finely divided precipitate of protein. Add 6 ml. of the petroleum ether and shake vigorously for ten minutes. Pour the emulsion thus produced into a centrifuge tube, cork, and spin at a low speed for about one minute. Pipette off as much as possible of the petroleum ether layer, taking care not to remove any of the watery layer with it.

**Determination of the Carotenes**

Place the petroleum ether extract in the colorimeter cup and read directly, using a violet filter, or transmission at 440 millimicrons. Use petroleum ether as blank or to set the instrument. Prepare a standard curve from a stock solution containing 50 mg. β-carotene per 100 ml. of petroleum ether, diluted 1 in 50 to give a standard solution for use containing 1 mg. per 100 ml. Use this to set up a series of standards as follows :

| Micrograms carotenes per 100 ml. serum | 0 | 50 | 100 | 200 | 400 | 600 |
|---|---|---|---|---|---|---|
| Ml. of standard solution (1 mg. per 100 ml.) | 0 | 0·25 | 0·50 | 1·0 | 2·0 | 3·0 |
| Ml. petroleum ether | 10 | 9·75 | 9·50 | 9·0 | 8·0 | 7·0 |

The concentration of carotenes in micrograms per 100 ml. of serum is read directly from this curve. The fact that 2 ml. of petroleum ether contain the carotenes from 1 ml. serum has been taken into account.

**Note.** Carotenes do not keep well since they easily oxidize. They then give a less yellow coloured solution in the petroleum ether. Even freshly bought specimens are not always satisfactory. The

author has found the carotenes supplied by Roche Products Ltd. to be reliable.

INTERPRETATION

The normal range may be taken as 50 to 200 μg. per 100 ml., though upper limits a little higher have been given and Campbell and Tonks (1949) give a lower limit of 20 μg. per 100 ml. (see Krebs and Hume, 1949). The blood level is greatly influenced by the amount of carotenes in the food. They almost disappear from the blood in a few weeks if absent from the diet. On the other hand an increase in serum carotenes is found if large amounts of carotene-rich vegetables are consumed. This usually means large quantities of carrots. Serum carotenes may then reach and exceed 500 μg. per 100 ml. This condition, *carotinæmia*, is accompanied by a yellow skin pigmentation and has already been referred to when jaundice was discussed (see p. 231). Increased values for serum carotenes have been reported in myxœdema, diabetes mellitus, and chronic nephritis.

**Determination of Vitamin A**

Measure 4 ml. of the petroleum ether solution, prepared as above, into one of the colorimeter tubes and evaporate off the solvent by placing in a water bath at 40° to 50° C. preferably passing a stream of dry carbon dioxide through the tube during the evaporation. Dissolve the residue in 0·5 ml. of chloroform and add a drop of acetic anhydride to remove traces of water which might cause cloudiness when the Carr Price reagent is added. With the colorimeter adjusted to chloroform, or with chloroform as blank, using an orange filter or transmission at 620 millimicrons, and with the tube in position, add quickly 3 ml. of Carr Price reagent from a pipette with a wide tip. The colour develops rapidly reaching a clearly defined maximum in five to fifteen seconds, and then fades quickly. Note this maximum reading of the colorimeter. A rather more stable colour is obtained if the Carr Price reagent is cooled to between 0° and 5° C. before use.

It is important that the glassware used should be perfectly dry. This also applies to the chloroform and the Carr Price reagent. When small amounts of water are present it may be impossible to read the colour. If any cloudiness develops the test must be repeated.

A correction has to be made for the colour given by the carotenes present. Prepare two standard curves for the colorimeter, one of standard amounts of vitamin A allowed to react with Carr Price reagent, the other of standard amounts of carotenes similarly treated. Then, when the serum carotene level has been determined, the amount of colour due to this is read from the carotene curve and subtracted from the actual reading of the colorimeter. The colorimeter reading due to the vitamin A is thus obtained and the serum vitamin A can be read from the vitamin A standard curve. This applies so long as 4 ml. of the petroleum ether extract is taken for the vitamin A determination. If a smaller volume has to be used, multiply the colorimeter reading by 4 divided by the volume of extract used.

(*a*) Vitamin A standard curve. Prepare a solution containing 400 μg. vitamin A per 100 ml. of chloroform and set up a series of tubes:

| Micrograms vitamin A per 100 ml. serum | 0 | 10 | 20 | 40 | 60 | 80 | 100 |
|---|---|---|---|---|---|---|---|
| Ml. solution of vitamin A (400 μg. per 100 ml.) | 0 | 0·05 | 0·1 | 0·2 | 0·3 | 0·4 | 0·5 |
| Ml. chloroform | 0·5 | 0·45 | 0·4 | 0·3 | 0·2 | 0·1 | 0 |

Add 3 ml. Carr Price reagent and read as described.

(*b*) Correction curve for the carotenes present. Dilute 1 ml. of the standard solution of carotene containing 50 mg. per 100 ml. chloroform, to 25 ml. with chloroform, thus obtaining a solution containing 2 mg. per 100 ml. Set up a series of tubes

| Micrograms carotene per 100 ml. serum | 0 | 100 | 200 | 300 | 400 | 500 |
|---|---|---|---|---|---|---|
| Ml. carotene solution (2 mg. per 100 ml.) | 0 | 0·1 | 0·2 | 0·3 | 0·4 | 0·5 |
| Ml. chloroform | 0·5 | 0·4 | 0·3 | 0·2 | 0·1 | 0 |

Add 3 ml. Carr Price reagent and read as described.

*Other Method.* Sobel and Snow (1947) used the colour given with activated glycerol dichlorhydrin. This colour is more stable than that given by the Carr Price reagent and traces of water present do not interfere.

INTERPRETATION

The normal serum vitamin A has been given as

19 to 36 μg. per 100 ml. (Krebs, 1950 ; Krebs and Hume, 1949).
21 to 45 μg. per 100 ml. (Sinclair, 1947).
21 to 48 μg. per 100 ml. (Campbell and Tonks, 1949).
22 to 47 μg. per 100 ml. (Yudkin, 1941).

All these ranges were obtained in England. Higher figures have been given. Thus

33 to 63 μg. per 100 ml. (Haig and Patek, 1942, in the U.S.A.).
30 to 90 μg. per 100 ml. (Lindquist, 1938, in Scandinavia).

It may be assumed that there is no deficiency when the serum level is above 20 μg. per 100 ml. When the concentration falls below about 10 μg. per 100 ml. dark adaptation begins to be impaired. The absorption of vitamin A from the intestine is defective when there is diminished fat absorption, and there is also increased loss in the fæces when liquid paraffin is being taken. Reduced blood levels may be found in liver disease when there is impaired formation of vitamin A from carotenes. The capacity to store vitamin A may also be affected in liver disease.

**Vitamin A tolerance tests** have been used to study vitamin A absorption in steatorrhœa. A large dose of the vitamin is given and the rise in the serum level studied. Thus Sinclair (1947) determines

serum vitamin A fasting, and four and seven hours after giving 10,000 I.U. of the vitamin per kilogram of body weight. Barnes, Wollaeger and Mason (1950), give 60,000 I.U. daily for four days before the test, and then for the test, 7,500 I.U. per kilogram, and determine the serum level at 0, 2, 4, 6, 9, and 24 hours. In normal patients a maximum of 2,000 to 3,000 I.U. per 100 ml. is reached in about four hours, after which it falls steadily. Absorption is impaired in steatorrhœa so that a much smaller rise is found. Kagan, Thomas, Jordan and Abt (1950) in studies on children with nephrosis used 6,000 I.U. per lb. of body weight and determined serum vitamin A at 0, 3, 6, and 24 hours.

**Note.** One International Unit (I.U.) of vitamin is defined as having the activity of 0·6 $\mu$g. of pure $\beta$-carotene. In the above figures it has been assumed that vitamin A has twice the activity of $\beta$-carotene so that 1 I.U. corresponds to 0·3 $\mu$g. of the vitamin.

## VITAMIN $B_1$, ANEURIN, THIAMINE

As regards biochemical laboratory methods two main approaches have been made to the problem of detecting a deficiency of vitamin $B_1$, the estimation of pyruvic acid in blood and of the vitamin itself in urine. There is only a small amount of thiamine in blood and a simple method for its estimation in routine work is not yet available. Friedemann and Kmieeack (1943) have used a thiochrome method. Microbiological methods have been employed, using the fungus Phycomyces blakesleeanus, which requires thiamine for its growth (see Meiklejohn, 1937 ; and Sinclair, 1938, 1939), and a Lactobacillus fermenti (Sarett and Cheldelin, 1944 ; Cheldelin, Bennett and Kornberg, 1946).

### Determination of Blood Pyruvic Acid

The estimation is based on the formation of the 2·4 dinitrophenylhydrazone, which reacts with strong alkali to form a reddish compound which can be estimated colorimetrically. Similar hydrazones of acetone and acetoacetic acid react in the same way but the pyruvic acid hydrazone can be separated from excess of added dinitrophenylhydrazine, and from the hydrazones of most other aldehydes and ketones which may be present, by extraction with sodium carbonate. Interference by acetoacetic acid can be overcome by allowing the protein filtrate to stand overnight. Oxaloacetic acid and ketoglutaric acid behave similarly, giving red colours.

The method was applied to blood by Lu (1939) and modifications were introduced by Bueding and Wortis (1940). In their method, after the protein filtrate had been allowed to react with the 2·4 dinitrophenylhydrazine, three extractions were made with ethyl acetate and the combined extract then re-extracted three times into 10 per cent. sodium carbonate. Friedemann and Haugen (1943) have simplified the procedure considerably and their technique is given.

### Method of Friedemann and Haugen

**Reagents.** 1. Sodium iodoacetate, 27 per cent. (25 per cent. iodoacetic acid). Adjust a 50 per cent. solution of iodoacetic acid in water to a *p*H of 7·8 with sodium hydroxide and dilute to 25 per cent. concentration of iodoacetic acid (see note 3).

2. Trichloracetic acid, 10 per cent. (w/v) solution. Prepare at frequent intervals and keep in the refrigerator when not in use. If allowed to stand at room temperature increased blanks may be obtained. A 10 per cent. metaphosphoric acid solution freshly prepared weekly can be used and has some advantages, but metaphosphoric acid is not as convenient a substance to use as trichloracetic acid.

3. 2·4-dinitrophenylhydrazine. Grind 100 mg. in a mortar with increasing small volumes of approximately 2 N hydrochloric acid until 100 ml. have been added. Filter and keep in the refrigerator when not in use.

4. Toluene, xylene or benzene. Under the conditions of the test these are more specific for pyruvic acid than ethyl acetate or ethyl ether, which give more nearly total keto-acids.

5. Sodium carbonate, 10 per cent. solution. Filter if not clear. Keep either in paraffin-lined bottles or in Pyrex vessels.

6. Approximately 1·5 N and 2·5 N solutions of sodium hydroxide. Keep in the same way as the carbonate.

7. Stock standard solution of pyruvic acid. Prepare 100 ml. of solution containing 100 mg. pyruvic acid, adding sulphuric acid to make the final solution N/10. Use either pyruvic acid freshly distilled at 16 mm. Hg, or lithium pyruvate (see Wendel, 1931–32). This solution is said to keep for several months in the refrigerator.

**Technique.** Withdraw the blood using a minimum amount of stasis, removing the tourniquet immediately after inserting the needle in the vein. The patient should not open and clench the hand. Do not use warm syringes. Use a 2 ml. or 5 ml. syringe, with a 21 gauge hypodermic needle, and eject the blood in a fine stream into five times its volume of a cold solution of trichloracetic acid placed in a cork-stoppered 15 or 50 ml. centrifuge tube. Stopper the tube, shake, and centrifuge. Keep in the refrigerator until ready to continue the estimation. This has been found to keep two days in the cold without loss of pyruvic acid. If patients are suffering from ketosis, allow the precipitated specimen to stand in the refrigerator overnight to eliminate the effect of acetoacetic acid. In any case this is likely to be small.

If the blood cannot be placed straight into the trichloracetic acid it can be kept for a short time, up to thirty minutes, by means of iodoacetate (or stabilized by the method of Long given below, note 3). Place 5 to 10 ml. of blood into a tube containing anticoagulant and 0·1 ml. of 27 per cent. sodium iodoacetate. Then proceed as above.

Pipette 3 ml. of the cold clear supernatant fluid obtained by centrifuging, into a 6 × $\frac{3}{4}$ inch test tube. Warm the contents of the

tube for ten minutes in a water bath at approximately 25° C. Add 1 ml. of dinitrophenylhydrazine reagent and allow to react at 25° C for exactly five minutes. For pyruvic acid add 3 ml. of solvent, xylene, toluene, or benzene, and pass a rapid stream of nitrogen (or air) through for two minutes. Remove most of the lower aqueous layer by means of the capillary pipette through which the gas has passed. Rotate the tube to dislodge solution adhering to the walls and remove any remaining solution. Add exactly 6 ml. of 10 per cent. sodium carbonate to the extract and again pass a rapid current of gas for two minutes. Remove the capillary and allow the two layers to separate. Insert a 5 ml. pipette quickly through the upper layer, blow a small amount of air through to clear the solvent, and pipette off 5 ml. of the carbonate extract, wiping the tip of the pipette free from any solvent before adjusting to the mark. Add 5 ml. of 1·5 N sodium hydroxide and mix. Read in the photoelectric colorimeter five to ten minutes after adding the alkali.

Carry out in the same way a reagent blank, and prepare a standard curve using a pyruvic acid solution obtained by diluting the stock standard 5 ml. to 100 ml.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Mg. pyruvic acid per 100 ml. blood . | 0 | 0·5 | 1·0 | 1·5 | 2·0 | 2·5 | 3·0 |
| Ml. pyruvic acid standard (1 ml.=0·05 mg.) | 0 | 0·5 | 1·0 | 1·5 | 2·0 | 2·5 | 3·0 |
| Ml. distilled water . . . . | 5·0 | 4·5 | 4·0 | 3·5 | 3·0 | 2·5 | 2·0 |

Dilute 1 volume of each with 5 volumes of acid, take 3 ml. and proceed as described above.

Use a green filter, transmission at 520 millimicrons.

**Notes.** 1. Friedemann and Haugen also used extraction with 8 ml. of ethyl acetate and state that this is useful for the determination of total keto-acids since almost all the α-ketoglutaric and oxaloacetic acids are extracted. Using toluene, xylene or benzene, only 10 per cent. of the α-ketoglutaric acid and 5 per cent. of the oxaloacetic acid are included. By the use of different filters the authors get approximate values for the concentration of each of these keto-acids.

2. The method can be applied to *urine*. Collect a twenty-four-hour specimen in a large cold bottle containing 5 ml. of 20 N sulphuric acid. If a single specimen is taken add 0·5 ml. per 100 ml. Storage up to twenty-four hours in the refrigerator is satisfactory. Add 2 ml. of urine to 10 ml. of 10 per cent. trichloracetic acid. Kept cold this will keep at least twenty-four hours. Treat the acidified urine with Lloyd's reagent, 0·75 gram to each 10 ml. Filter immediately. Then proceed with 3 ml. of this filtrate as described for 3 ml. of the protein-free filtrate from the blood.

3. *Stabilization of Blood for the Determination of Pyruvic Acid.* The changes in blood pyruvic acid which occur when blood is kept have been studied by Long (1944). There is first a fall in pyruvic acid which is more marked if oxalate is used as anti-coagulant than with citrate. With the former as much as 75 per cent. may be lost in thirty minutes whereas with the latter only some 25 per cent. Later,

in a matter of days, the pyruvic acid rises to a concentration several times greater than that originally present. Long supposes that in the first case pyruvate reacts with triosephosphate to give lactate and phosphoglycerate, whilst in the second pyruvate is formed from phosphoglycerate. Oxalate and fluoride inhibit the latter but not the former, citrate neither, so that the two reactions partly counteract each other. Iodoacetate prevents the conversion of pyruvate to lactate. Bueding and Wortis found that a small increase in pyruvate, from 3 to 20 per cent., occurred within thirty minutes when iodacetate was used.

Long showed that pyruvate could be completely stabilized by hæmolysing the blood with cetavlon (cetyl trimethyl ammonium bromide) and preserving with sodium fluoride at a *p*H of 4. His method is as follows : dissolve 168 mg. of citric acid monohydrate in about 0·5 ml. of distilled water and add 40 per cent. sodium hydroxide until a *p*H of 4 is reached. Pipette half of this citrate buffer into a test tube, evaporate to dryness *in vacuo* on a boiling water bath. Add 21 mg. of sodium fluoride and 20 mg. of cetavlon (75 per cent.). Stopper with a white rubber bung. There is no deterioration in the contents for several weeks. Long (1946) showed that this also prevents any change in the lactic acid content of the blood.

INTERPRETATION

The normal range for blood pyruvic acid is from 0·5 to 1·0 mg. per 100 ml. In marked deficiency of vitamin $B_1$ values up to 2 to 3 mg. per 100 ml. are found. This increase is due to the fact that thiamine, phosphorylated as cocarboxylase, forms the prosthetic group of carboxylase, which catalyses the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide. Increases in pyruvic acid have also been reported in diabetes mellitus (see p. 81), in congestive heart failure, in diarrhœa and other digestive disturbances, and in some acute infections.

Bueding, Stein and Wortis (1941) suggested using the increase in blood pyruvic acid which results from taking glucose. Such an increase is found in normal persons, but reaches a maximum in an hour and the blood pyruvic acid returns to normal limits within three hours. In thiamine deficiency a greater increase occurs and a longer time is required for the return to normal. This test is said to show abnormal results before the fasting blood pyruvic acid is increased. Williams *et al.* (1943) gave 0·4 mg. of glucose per kilogram of body weight, intravenously as a 50 per cent. solution. Alternatively they gave the glucose by mouth as in the Exton Rose test (p. 51). Blood for determination of pyruvic acid was taken fasting, and half, one, and two hours after giving glucose. The glucose can be given as in the glucose tolerance test, p. 45, that is, 50 grams in a cupful of water with the patient fasting. The rise in pyruvic acid should not produce a blood level of more than about 1·2 mg. per 100 ml. If this is exceeded there is evidence in favour of a vitamin $B_1$ deficiency.

Bisulphate binding substances in blood have also been determined but this is not as satisfactory as the estimation of pyruvic acid.

**Determination of Lactic Acid.** Method of Barker and Summerson (1941)

It will be convenient to include this whilst pyruvic acid is being discussed, in view of their close connection. In the method given, protein-free filtrate is treated with copper sulphate and solid calcium hydroxide to remove glucose. A portion of the filtrate from this process is treated with sulphuric acid to convert the lactic acid into acetaldehyde, the amount of which is measured by means of the colour given by *p*-hydroxydiphenyl.

**Reagents.** 1. Copper sulphate, 20 per cent. solution of $CuSO_4$, $5H_2O$.

2. Copper sulphate, 4 per cent. solution.

3. Powdered calcium hydroxide.

4. Concentrated sulphuric acid.

5. *p*-hydroxyphenol, 1·5 per cent. in 0·5 per cent. sodium hydroxide.

6. Standard solution of zinc or lithium lactate. Dissolve 0·213 gram of pure dry lithium lactate in about 100 ml. of water, add 1 ml. of concentrated sulphuric acid and make up to a litre with water. Dilute 1 in 20 to obtain a standard containing 10 μg. per ml.

**Technique.** Change in lactic acid content of the blood can be prevented by the method of Long used for pyruvic acid. Remove protein from the blood by any of the methods in common use, for example, trichloracetic acid, tungstic acid, zinc hydroxide. Take a volume of protein-free filtrate containing between 20 and 100 μg. of lactic acid. In the case of most patients this requires a volume equivalent to about 0·2 ml. of blood. Add this to 1 ml. of 20 per cent. copper sulphate solution and make up to 10 ml. with water. Add about 1 gram of powdered calcium hydroxide and shake vigorously. Stand at room temperature for at least half an hour, shaking occasionally, and then centrifuge. Pipette 1 ml. of supernatant fluid into a test tube 18 to 23 mm. internal diameter, taking care not to include any solid material which may be present in the surface film. Add 0·05 ml. of 4 per cent. copper sulphate solution and run in exactly 6 ml. of concentrated sulphuric acid from a burette, mixing well while doing so. Then place the tube upright in a boiling water bath for five minutes. Remove, cool to below 20° C. in cold water, and add exactly 0·1 ml. of the *p*-dihydroxyphenyl. Disperse the precipitated reagent quickly through the solution and place the tube in a bath at 30° C. for thirty minutes, redispersing the precipitated reagent at least once during this time. Place in a boiling water bath for ninety seconds, cool to room temperature, and read in the photoelectric colorimeter with transmission at 560 millimicrons, or using a yellow-green filter. Put up a reagent blank using water instead of protein-free filtrate, and a standard using 5 ml. of the standard lactate solution, both carried through in the same way as the test.

**Calculation.** Since 5 ml. of standard contains 50 μg. (0·05 mg.), one-tenth of which is used for developing the colour, and since the colour developed in the determination corresponds to 0·02 ml. of blood,

Mg. lactic acid per 100 ml. blood

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}005 \times \frac{100}{0{\cdot}02}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 25.$$

**Note.** Long (1946) has published a method for determining lactic acid in which, after removing proteins by means of trichloracetic acid, the lactic acid is oxidized by ceric sulphate to acetaldehyde. This is absorbed in sodium bisulphite and subsequently titrated with iodine.

INTERPRETATION

The normal resting range for blood lactic acid is 6–18 mg. per 100 ml. Exercise causes a considerable increase. Since the liver converts lactic acid to glycogen there is an increase in liver disease, but this only becomes appreciable when there is marked failure of liver function. Stotz and Bessey (1942) have studied the ratio of pyruvic acid to lactic acid. In normal persons this remains relatively constant with varying levels of lactic acid, but is increased when there is a deficiency in vitamin B.

**Determination of Thiamine in Urine by the Thiochrome Reaction.** Method of Johnson *et al.* (1945).

Thiamine is converted to thiochrome by the action of potassium ferricyanide and the degree of fluorescence in isobutyl alcohol is measured.

**Reagents.** 1. Activated Decalso or Permutit. There is considerable variation between different samples. A product is required which will adsorb thiamine well and settle out rapidly. When about 200 mg. are shaken with water in a 10 ml. measuring cylinder all the particles should settle rapidly to the bottom in not more than two minutes. To obtain a suitable product, suspend material which will pass a 100-mesh sieve, in 1 per cent. acetic acid in distilled water in large cylinders. Separate the granules which settle down to the bottom in two minutes from the lighter particles, by decanting. Boil these three times with 1 per cent. acetic acid with settling and decanting between fresh additions of acetic acid. Wash the product with distilled water and dry at 110° C. If addition of potassium ferricyanide results in the formation of Prussian blue, wash the product in a warm, fairly strong, solution of hydrochloric acid. Carry out test runs with synthetic thiamine. Unsatisfactory products can be reactivated by the method of Najjar and Wood, 1940. The activity of a satisfactory product remains constant for months if it is kept dry.

2. Approximately 25 per cent. potassium chloride solution.

3. Sodium hydroxide, 15 per cent. solution.

4. Potassium ferricyanide, 0·25 per cent. solution, prepared fresh daily.

5. Isobutyl alcohol or *n*-butyl alcohol. This should give almost no blank. Test each new batch before use. If desired collect after use and recover by redistillation.

6. Dilute acetic acid, approximately 1 per cent. v/v in distilled water.

**Technique.** Collect urine in glass vessels and store in amber bottles, adding 100 mg. of oxalic acid for every 25 ml. of urine. Measure 2 ml. of urine (0·5 ml. if the result is likely to be high) into a glass-stoppered test tube, or small measuring cylinder, and add about 200 mg. of the activated Decalso or Permutit and mix with ten rapid shakes. The adsorption is not critically affected by the acidity so long as the *p*H is between 3 and 6 which is assured by use of the oxalic acid. Now add about 8 ml. of the dilute acetic acid, close the tube with a clean finger and mix by inversion ten times. Stand for a short time and remove the supernatant fluid. Add 8 ml. of acid and repeat the process of standing, inversion and removal of supernatant fluid. This washing is important since if it is not effective the final fluorescence has a silvery-blue admixture to the true thiochrome mauve, thus giving unsatisfactory readings. The thiamine is firmly adsorbed so that repeated washings only remove interfering substances. If any trouble is experienced in this connection, further washings are made.

Add 0·5 ml. of potassium chloride solution and shake gently, taking care to avoid splashing the solid far up the side. Elution is complete within thirty seconds. Add 0·1 ml. of 0·25 per cent. potassium ferricyanide and 0·25 ml. of 15 per cent. sodium hydroxide, shaking gently after each addition. Add 2 ml. of isobutyl alcohol, insert the stopper, and shake vigorously for about a minute (twenty-five shakes up and down). Stand to allow separation of the two phases, or centrifuge for a short time. Transfer 1 ml. of the supernatant fluid to a small test tube and match the colour in a visual comparator, in ultra-violet light, with standards prepared from solutions of thiamine hydrochloride treated in the same way.

A convenient range covers 0 to 100 μg. per 100 ml. Prepare a solution of 40 mg. thiamine hydrochloride in 100 ml. of water and dilute 5 ml. to 100 ml., adding 400 mg. oxalic acid, to obtain a solution containing 200 μg. per 100 ml. Suitable standards are 0, 10, 25, 50, 75, 100, μg. per 100 ml.

INTERPRETATION

The excretion of thiamine is greatly affected by variations in the amount taken in the diet. Further variations result from different methods used in its estimation. Daily excretion would appear to range from 50 to 500 μg., but while some workers have reported even lower figures, an excretion of under 90 μg. has been regarded as evidence of a deficient intake (Melnick, 1942). The Oxford Nutrition survey used below 5 μg. per 100 ml. as abnormal and below 2 μg. as showing extreme abnormality.

More reliable information has been held to be given by studying the amount excreted after a test dose. Several variants have been used. Melnick and Field (1942) injected intramuscularly a dose based on 0·35 mg. per square metre of surface area, and collected urine for the four-hour period following the injection. At least

50 μg. (about 8 per cent. of the test dose) should be excreted in that time. Najjar and Holt (1940) injected 1 mg. of thiamine intravenously and obtained at least 110 μg. in the following four hours, in adults. Mason and Williams (1942) give 1 mg. by mouth (0·4 mg. per 1,000 calories) and get 100 μg. in the urine in twenty-four hours.

These tests are more sensitive than the estimation of blood pyruvic acid. Diminished excretion of thiamine following a test dose gives the earliest indication of a deficiency of $B_1$. Simple estimation of pyruvic acid is the least sensitive, the increase in pyruvic acid after administration of glucose being intermediate between these.

Little use has been made of chemical methods for investigating deficiencies of vitamins other than A, $B_1$, and C unless one includes the use of serum alkaline phosphatase in rickets and prothrombin time in connection with vitamin K. However, relatively simple methods are available for the estimation of riboflavin (vitamin $B_2$) and $N^1$-methylnicotinamide, an excretion product of nicotinic acid, in urine.

## RIBOFLAVIN

**Determination of Riboflavin in Urine** (Najjar, 1941 ; Johnson *et al.*, 1945 ; see also, Slater and Morrell, 1946)

The riboflavin is extracted with an acetic acid-pyridine-butyl alcohol mixture after interfering urinary pigments have been oxidized with permanganate. The concentration of riboflavin is then measured fluorimetrically.

**Reagents.** 1. Dry powdered oxalic acid.

2. A mixture of equal volumes of pyridine and glacial acetic acid.

3. Potassium permanganate, 4 per cent. solution.

4. Hydrogen peroxide, 3 per cent. (10 volumes per cent.).

5. Isobutyl alcohol or *n*-butyl alcohol. Examine each batch before use before the ultra-violet light. Redistil if there is perceptible fluorescence.

6. Anhydrous sodium sulphate.

**Technique.** Collect a twenty-four-hour specimen as described under thiamine, using oxalic acid and keeping in an amber bottle. Carry out all operations in a diffuse light, avoiding direct sunlight. Measure 0·5 ml. of urine into a 10 ml. glass-stoppered test tube and add 0·5 ml. of the pyridine-acetic acid mixture. Add a drop of potassium permanganate solution and shake gently for about a minute. Then add 2 drops of hydrogen peroxide and again shake gently. The permanganate should be destroyed within a few seconds, if not, add a further drop of hydrogen peroxide and warm slightly to 70° F. Add 1·5 ml. of isobutyl alcohol, stopper, and shake vigorously up and down twenty-five times, that is, for about a minute. Stand to allow the layers to separate, add a small amount of anhydrous sodium sulphate, rotate between the hands to clear the alcohol layer, and stand a minute or two for the layers to separate. Transfer 1 ml. of the clear upper alcohol layer to a thin-walled Pyrex

tube, 75 × 10 mm., and compare with standards in ultra-violet light. The alcohol solutions remain stable for at least two hours. A silvery blue fluorescence invariably present can be absorbed by a suitable yellow filter. To get a true riboflavin reading, after the initial reading has been made, expose to strong ultra-violet light for exactly an hour to destroy the riboflavin and read again. The amount of fluorescence not destroyed by ultra-violet light is, however, remarkably constant, being about half the initial reading in most urines, and much less, about one-fifth, in urines collected after a test dose of riboflavin has been taken.

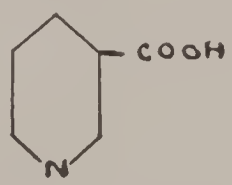
Prepare standard solutions in the same way as described for thiamine. In this case a suitable range is from 0 to 100 μg. per 100 ml. Develop fluorescence from these in the same way as for the urine which is being tested.

INTERPRETATION

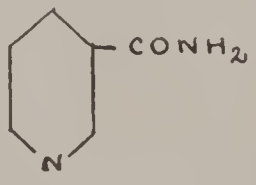
The daily excretion is greatly influenced by the amount taken in the diet. On a satisfactory intake it is between 0·5 and 0·8 mg. Tests using a standard dose are more satisfactory than an estimation made on a twenty-four hour specimen. If 16 μg. per kilogram body weight are given intravenously at least 25 per cent. of the amount given should be excreted during the following four hours (see Najjar and Holt, 1941 ; Axelrod *et al.*, 1941).

## NICOTINIC ACID, NIACIN

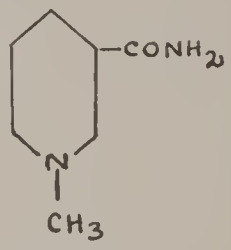
Nicotinic acid, a deficiency of which leads to pellagra, is present in two important co-enzymes, co-enzymes I and II which are found in almost all cells and are required for a number of important oxidation processes. It is also present in food as the amide, nicotinamide.




Nicotinic Acid




Nicotinamide




N¹-methylnicotinamide

Nicotinic acid can be determined colorimetrically using the reaction with cyanogen bromide and an aromatic amine. Aniline has been used. Rosenblum and Joliffe (1940) used metol, Wang and Kodicek (1943) *p*-aminoacetophenone. Any nicotinamide is first hydrolysed to convert it to nicotinic acid.

However, nicotinic acid is also excreted as metabolites. The most important of these is N¹-methylnicotinamide, earlier known as $F_2$. This is not determined by the colorimetric method for nicotinic acid

referred to above. The amount of this excreted in the urine appears to give a better indication in regard to nicotinic acid nutrition than does excretion of the vitamin itself. $N^1$-methylnicotinamide fluoresces in alkaline solution so that it can be determined fluorimetrically after preliminary adsorption, elution and extraction, as described below. The method of Johnson *et al.* is given. See also Hochberg *et al.* (1945), Huff and Perlzweig (1947), and for further information concerning other metabolites of nicotinic acid, Jones (1951).

**Determination of $N^1$-methylnicotinamide in Urine** (Johnson *et al.*, 1945)

The $N^1$-methylnicotinamide is adsorbed at a *p*H of 4·5 on to a column of synthetic zeolite, eluted with potassium chloride, made alkaline and extracted with *n*-butyl alcohol. It is then compared fluorimetrically with standards.

**Reagents.** These are the same as for thiamine in urine.

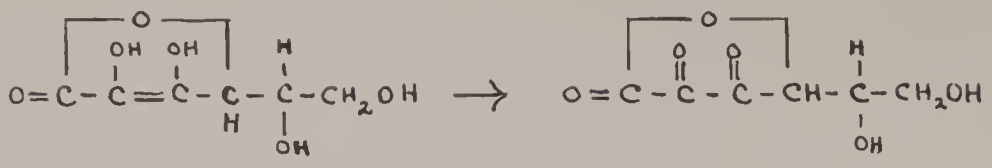
**Technique.** Collect urine as for thiamine, and proceed as described for thiamine, up to the point of washing the Permutit or Decalso on to which the adsorption has been carried out. Whereas thiamine is firmly adsorbed, this is not so for $N^1$-methylnicotinamide, which is washed off relatively easily. So wash only twice and adhere closely to a definite technique for test and standards. After elution with 0·5 ml. of potassium chloride, run 2 ml. of isobutyl alcohol into the tube, add 0·25 ml. of 15 per cent. sodium hydroxide, stopper at once and shake up and down for about a minute (twenty-five times). Stand to separate, or centrifuge. Transfer 1 ml. of the supernatant alcohol layer to a small test tube and compare in the ultra-violet light with standards similarly treated. This comparison should not be made earlier than five minutes after shaking with the sodium hydroxide. For the standards a range of 0 to 10 mg. of $N^1$-methylnicotinamide per 100 ml. is convenient. No blank correction is required so long as the urine level is below 1·5 mg. per 100 ml.

INTERPRETATION

The daily excretion of $N^1$-methylnicotinamide, when adequate nicotinic acid is being taken, ranges from 3–17 mg. (Huff and Perlzweig, 1947), the average being 7 mg.

## ASCORBIC ACID

There are some points concerning the chemistry of ascorbic acid which are important in connection with its determination. It forms white crystals which are stable in air, but it oxidizes easily in solution being converted into dehydroascorbic acid. Both these are biologically active, since dehydroascorbic acid can be reduced in the body to *l*-ascorbic acid. The rate of oxidation of *l*-ascorbic acid increases with increasing *p*H, so that it is much more stable in acid solution. Dehydroascorbic acid itself undergoes further oxidation above *p*H 5,



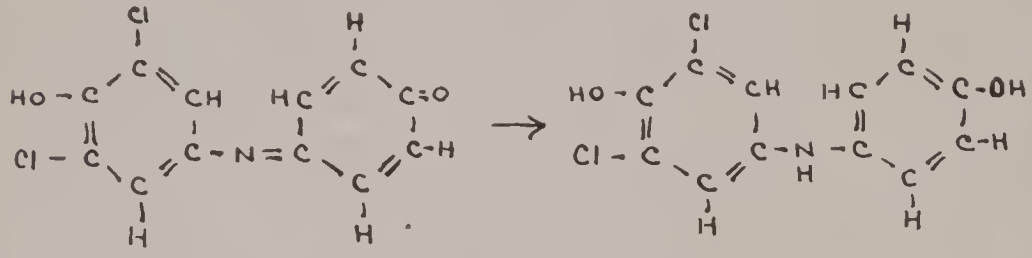
*l*-ascorbic acid dehydroascorbic acid

the ring opening to give diketogulonic acid with subsequent breakdown to oxalic acid and *l*-threonic acid,

$$HOOC.CHOH.CHOH.CH_2OH.$$

With opening of the ring biological activity ceases.

One of the methods most frequently used for the determination of ascorbic acid is the titration of 2·6 dichlorophenolindophenol in acid solution. This blue compound is red in acid solution, and on titration with a solution of ascorbic acid is reduced to the colourless leucobase, the ascorbic acid being oxidized to dehydroascorbic acid. It is thus



2·6 dichlorophenolindophenol colourless leucobase

seen that if a mixture of *l*-ascorbic acid and dehydroascorbic acid is present, only the former reacts with the dye, although both are biologically active. Dehydroascorbic acid, however, can be reduced back to *l*-ascorbic acid in acid solution by hydrogen sulphide, so that titration then measures all the biologically active substances present. In body fluids such as blood and urine only *l*-ascorbic acid appears to be present, so that if the estimation is carried out on a fresh specimen before conversion to dehydroascorbic acid is able to occur, all the active vitamin present will be measured. However, particularly in urine, other substances may be present, which react with the dichlorophenolindophenol, thus impairing the reliability of the method. Attempts have been made to overcome this and will be referred to later.

Another method suggested for the determination of ascorbic acid is to form the derivative of dehydroascorbic acid with 2·4-dinitrophenylhydrazine. When treated with sulphuric acid this gives a red product, which can be used for colorimetric estimation. In this case it is thus necessary to convert all the ascorbic acid into dehydroascorbic acid. This can be done by shaking with norit and filtering. The norit clears the liquid and oxidizes the ascorbic acid.

The oxidation of ascorbic acid by oxygen is catalysed by certain metallic ions, such as the cupric ion.

**Determination of Ascorbic Acid in Urine. Method using Titration of 2·6 dichlorophenolindophenol** (Harris and Ray, 1935).

**Reagents.** 1. Glacial acetic acid.

2. Solution of 2·6-dichlorophenolindophenol. Weigh out accurately 40 mg. of the dye and dissolve in 100 ml. of distilled water. One ml. of this solution is equivalent to 0·2 mg. of ascorbic acid. Since the keeping qualities of the solution are poor it is necessary to prepare it freshly at frequent intervals. It should not be kept in use for more than a week. It may be more convenient to use the tablets of the dye, which can be obtained from British Drug Houses. These contain an amount of dye equivalent to 1 mg. of ascorbic acid, so that when dissolved in 10 ml. of water they give a solution of which 1 ml. is equivalent to 0·1 mg.

Solutions of dye can be checked against an ascorbic acid solution of known concentration. Dissolve 40 mg. of pure dry ascorbic acid in 100 ml. of 10 per cent. acetic acid. Dilute 5 ml. of this to 100 ml. with 10 per cent. acetic acid. Titrate 0·5 ml. of the dye with this solution. Five ml. should be required to decolorize it.

Ascorbic acid itself can be standardized against iodine. Since 1 molecule of ascorbic acid loses two hydrogen atoms on oxidation, one molecular weight in grams of ascorbic acid is equivalent to twice the atomic weight in grams of iodine. So a normal solution of ascorbic acid contains 176/2, that is, 88 grams per litre, or 88 mg. per ml. Hence 1 ml. of N/100 iodine is equivalent to 0·88 mg. ascorbic acid, or

1 mg. ascorbic acid is equivalent to 1·14 ml. N/100 iodine.

Dissolve 50 mg. of the ascorbic acid in 100 ml. of glass-distilled water and titrate 5 ml. with N/100 iodine using starch as indicator. Use a microburette. Five ml. contains 2·5 mg. of ascorbic acid and so should require $1{\cdot}14 \times 2{\cdot}5 = 2{\cdot}85$ ml. of N/100 iodine.

**Technique.** Pipette 0·5 ml. of the dye into a test tube (6 × 1 inch) and add 1 ml. of glacial acetic acid. Run in the urine slowly, with constant shaking, until the red colour has been discharged. Note the amount of urine required.

**Calculation.** 0·5 ml. of dye = 0·1 mg. ascorbic acid. So, Mg. ascorbic acid per 100 ml. of urine

$$= \frac{0{\cdot}1}{\text{volume of urine required}} \times 100 = \frac{10}{\text{ml. urine required}}.$$

*Alternative Technique.* Dilute a portion of the urine with one-ninth its volume of glacial acetic acid and determine the volume required to decolorize 0·5 ml. dye. Then

Mg. ascorbic acid per 100 ml. urine = 11·1 ÷ ml. urine required. This ensures a uniform concentration of acetic acid.

**Notes.** 1. The urine must be titrated fresh, that is, within a few minutes of being passed, unless acetic acid is added immediately in the proportion of 1 part to 9 parts of urine. Such acidified urine will

keep for several hours. The determination is most often done on urine specimens collected as part of saturation tests (see below) which can be titrated at once. A rather large amount of acetic acid is required to protect twenty-four hour specimens, the estimation of ascorbic acid in which is not very convenient.

2. Other substances which also decolorize the dye are present in urine, so that the result may not be an accurate value for the ascorbic acid present. However, it is sufficiently accurate for most clinical purposes. Some modifications of the method have made use of the fact that the reaction of ascorbic acid with the dye is more rapid than that of other substances present which react similarly, but it is doubtful whether this difference is such as to warrant methods based on it. In saturation tests, because of the greater quantity of ascorbic acid often present, the effect of these interfering substances is much less important and can be ignored.

INTERPRETATION

The daily output of ascorbic acid is roughly half the intake. A usual amount is 20 to 30 mg. The daily intake of persons on a reasonably satisfactory amount of vitamin C would range from 30 to 80 mg. The minimum amount which protects against scurvy is 10 to 15 mg. daily. In states of vitamin C deficiency urine ascorbic acid is much reduced and probably disappears completely. The small amounts which appear to be measured by the above method are probably due to other reducing substances. The quantity of ascorbic acid excreted in twenty-four hours is not a satisfactory method of studying the nutritional condition in regard to vitamin C, even apart from the fact that preservation of such a specimen is troublesome.

## Ascorbic Acid Saturation Tests

These tests have been widely used. They are based on the assumption that if the previous intake of the vitamin has been insufficient, the tissues will take up quite large amounts when ascorbic acid is given so that little or none will be excreted in the urine. On the other hand, persons whose intake of ascorbic acid has been adequate will excrete appreciable amounts of such ingested ascorbic acid. Various dosages have been used, the ascorbic acid has been given orally and intravenously, and different times for urine collection have been used. Thus at least 200 mg. should be excreted in the four hours after intravenous injection of 500 mg. ascorbic acid given in 5 ml. of sterile distilled water. The test described is almost identical with that of Harris and Abbasy (1937).

**Technique.** A dosage of 70 mg. of ascorbic acid per stone of body weight (11 mg. per kilogram) is used, giving an average dose for adults of about 700 mg. This may be given in water during the morning. Only one specimen of urine is collected covering the period from four to six hours after taking the ascorbic acid, which is the time when excretion is at its maximum. Thus the test might be carried out as follows :

10 *a.m.* Give by mouth 70 mg. ascorbic acid per stone of body weight dissolved in about 4 to 6 oz. of water.
2 *p.m.* Empty the bladder completely and discard the urine.
4 *p.m.* Empty the bladder completely and estimate the ascorbic acid content almost immediately as described above.

Repeat the test daily until a normal response is obtained.

INTERPRETATION

In the case of persons in a normal nutritional state as regards vitamin C, an excretion of roughly 5 mg. per stone, that is an average of about 50 mg., should be obtained on the first or second day. When vitamin C deficiency is present, a longer time is required. In milder cases up to six to ten days may be needed; in severe cases, in which symptoms of scurvy may be present, up to two to three weeks may be necessary. In cases where a deficiency is present the titration corresponds to the presence of only a very few mg. of ascorbic acid, usually less than three, this probably being due to reducing substances other than ascorbic acid. This level is, as a rule, maintained until the deficiency has almost been made good when there is quite a quick rise to the region of 40 to 50 mg. for the two-hour urine found in a normal person. With such specimens of urine containing 50 mg. ascorbic acid in perhaps about 100 ml. urine, a more accurate result is obtained if the urine is diluted 1 to 5 or 1 to 10 with water, before carrying out the titration. Otherwise very small amounts of urine, less than 1 ml., will be required.

This test has been criticized quite considerably in recent years, but appears to be as convenient and satisfactory as anything available at present. Ascorbic acid in therapeutically useful amounts is being given whilst the test is carried out, and the effect may be visible clinically.

## Determination of Ascorbic Acid in Blood

### Determination of Plasma Ascorbic Acid by 2·6-dichlorophenolindophenol Titration

**Reagents.** 1. Trichloracetic acid, 10 per cent. (w/v) solution, *or* freshly prepared 5 per cent. metaphosphoric acid.

2. Solution of 2·6-dichlorophenolindophenol. Prepare as under urine so that 1 ml. is equivalent to 0·2 mg. ascorbic acid. Dilute this, 5 ml. to 25 ml. in a 25 ml. stoppered cylinder. One ml. of this solution is equivalent to 0·04 mg. ascorbic acid.

**Technique.** Mix equal volumes (4 ml. is convenient) of plasma separated immediately after withdrawing the blood, and of the trichloracetic or metaphosphoric acid. Filter or centrifuge. Pipette 0·2 ml. of the diluted dye solution into a test tube and titrate with the filtrate until the reddish colour has disappeared.

**Calculation.** 0·2 ml. dye is equivalent to 0·008 mg. ascorbic acid. Hence mg. ascorbic acid per 100 ml. plasma

$$= \frac{100}{\text{ml. titration}} \times 2 \times 0{\cdot}008 = \frac{1{\cdot}6}{\text{ml. titration}}.$$

Whilst metaphosphoric acid has advantages over trichloracetic acid, its keeping properties are poor, even in the solid form once the reagent bottle has been opened, so it is usually more convenient to use trichloracetic acid.

**Note.** If the plasma cannot be separated immediately, blood can be preserved by the method used by Mindlin and Butler (1938). The blood is collected into a test tube containing 1 drop each of 5 per cent. potassium cyanide and 20 per cent. potassium oxalate for 4 to 5 ml. of blood.

Mindlin and Butler have also used a method in which an excess of dye is added to plasma, and the amount of unreduced dye is determined colorimetrically.

INTERPRETATION

Plasma ascorbic acid levels are of doubtful value in diagnosing scurvy and subclinical conditions of vitamin C under-nutrition. Various levels from about 0·7 mg. per 100 ml. downwards have been stated to indicate scurvy, but while persons on an adequate intake of vitamin C will generally be found to have a plasma level between 0·6 and 2·0 mg. per 100 ml. with typical values from 0·8 to 1·4, it would appear that all values down almost to zero are obtained in apparently healthy persons. While it is possible that some of these figures may be due to defective methods of estimating the ascorbic acid, at the present time it is not possible to draw any reliable conclusions from plasma levels.

More recently attention has been directed to whole blood. It has been shown (Crandon *et al.*, 1940 ; see also Butler and Cushman, 1940 ; Butler *et al.*, 1943) that ascorbic acid disappears more rapidly from the plasma than from the cells when a diet low in vitamin C is taken. The largest concentration of ascorbic acid is found in the white blood cells and in the platelets which may contain as much as 25 to 30 mg. per 100 grams whereas the red cells may have 0·8 to 1·0 mg. per 100 grams. The white cells appear to be the last to be depleted.

Roe and Kuether have worked out a method for the determination of ascorbic acid in whole blood colorimetrically as the 2·4-dinitrophenylhydrazone in acid solution. This can be used for plasma and urine if desired.

**Determination of Whole Blood Ascorbic Acid.** Method of Roe and Kuether (1943)

**Reagents.** 1. Trichloracetic acid, 6 per cent. (w/v) solution.

2. 2·4-dinitrophenylhydrazine reagent. Dissolve 2 grams of the solid in 100 ml. of approximately 9 N-sulphuric acid (prepare this by adding 1 part of the concentrated acid to 3 parts of water). Filter when necessary and keep in the refrigerator.

3. Acid washed norit. Place 200 grams of norit in a large flask, add a litre of 10 per cent. hydrochloric acid, heat to boiling and filter

with suction. Transfer the cake of norit to a beaker, add a litre of distilled water, stir thoroughly and filter. Repeat until the washings give a negative test for ferric ions. Dry overnight at 110° to 120° C., but not higher. Some batches of norit do not need this washing. If a blank using norit washed with trichloracetic acid is the same as with trichloracetic acid alone then washing is unnecessary.

4. Sulphuric acid, 85 per cent. Add 900 ml. of the concentrated acid to 100 ml. of distilled water.

5. Thiourea solution. Dissolve 10 grams of thiourea in 100 ml. of a mixture of equal volumes of ethyl alcohol and water. This keeps for at least two months but should be checked occasionally. It should reduce potassium permanganate readily.

6. Standard solution of ascorbic acid. Prepare by dissolving 50 mg. in 100 ml. 4 per cent. trichloracetic acid. Dilute 5 ml. of this to 200 ml. with the trichloracetic acid solution. This solution contains 12·5 $\mu$g. per ml.

**Technique.** To 6 ml. of 6 per cent. trichloracetic acid in a centrifuge tube add 2 ml. of whole blood (or plasma) slowly, with constant stirring to produce a fine suspension. Stand for five minutes, centrifuge, and then add 0·3 gram of the acid-washed norit to the supernatant fluid. Cork and shake vigorously. Filter. This converts the whole of the ascorbic acid present into dehydroascorbic acid.

Measure out 2 ml. of the filtrate into each of two test tubes. Add 1 drop of thiourea solution to each. Keeping one tube as blank, add to the other 0·5 ml. of the 2·4-dinitrophenylhydrazine. Stopper and place in a water bath at 37° C. for exactly three hours. Remove and place both test and blank tubes in ice-cold water and add slowly 2·5 ml. of 85 per cent. sulphuric acid, drop by drop, and taking about half a minute to do so, so that there is no appreciable rise in temperature. Finally, add 0·5 ml. of the dinitrophenylhydrazine to the blank. Mix well the contents of both tubes while still in iced water. Remove to a rack, and after thirty minutes read in the photoelectric colorimeter at 540 millimicrons, or using a yellow-green filter. Read the ascorbic acid content on a standard curve, prepared as follows :

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Mg. ascorbic acid per 100 ml. blood | 0 | 0·25 | 0·5 | 0·75 | 1·0 | 1·25 | 1·5 | 2·0 | 2·5 |
| Ml. standard solution (12·5 $\mu$g. per ml.) | 0 | 0·4 | 0·8 | 1·2 | 1·6 | 2·0 | 2·4 | 3·2 | 4·0 |
| Ml. 4 per cent. trichloracetic acid | 8·0 | 7·6 | 7·2 | 6·8 | 6·4 | 6·0 | 5·6 | 4·8 | 4·0 |

Add norit, shake, stand, filter, take 2 ml. of the filtrate, and proceed as described above for the test.

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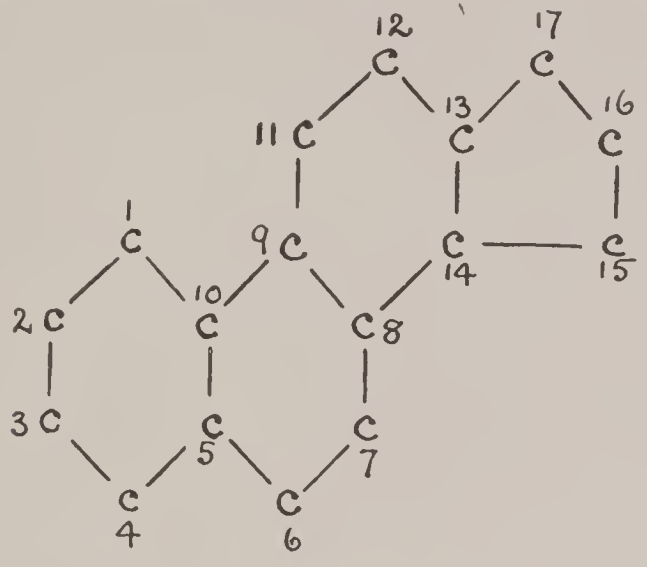
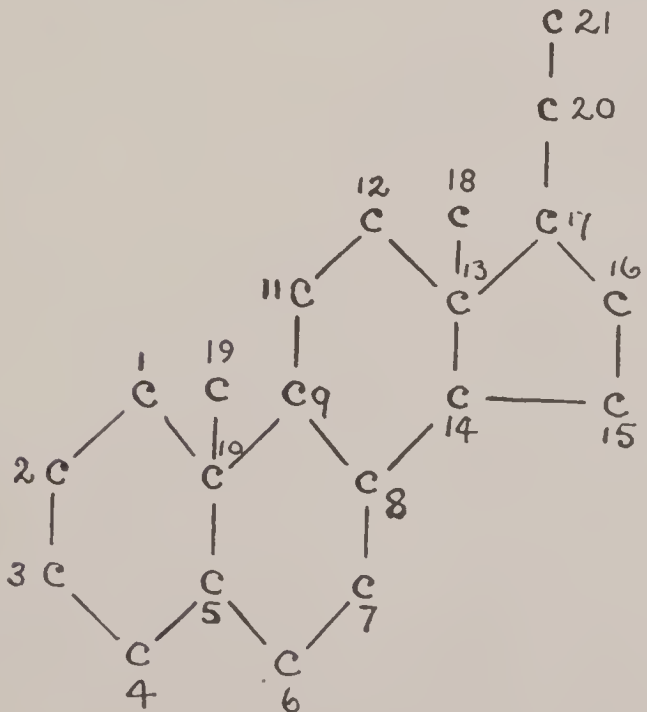
*CHAPTER XXIII*

# HORMONES

In this chapter the hormones considered will be the male sex hormones, androgens, produced by the testis and adrenal cortex : the female sex hormones, œstrogens produced by the ovary, and progesterone from the corpus luteum and placenta ; and corticosteroids, from the adrenal cortex. All these are steroids, that is, they are derivatives of the tetracyclic hydrocarbon, cyclopentanophenanthrane, $C_{17}H_{28}$, the carbon atoms of which are numbered as shown in formula I. Substituted groups are sometimes found on carbon




I



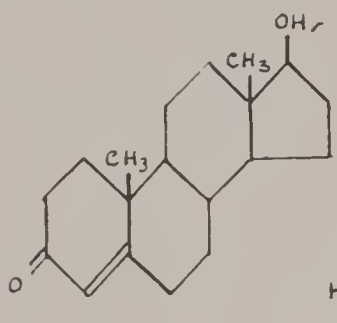

II

atoms 3, 10, 11, 13, and 17. Those which introduce further carbon atoms include methyl groups on 10 and 13, and a side chain of two carbon atoms on 17. The maximum number of carbon atoms is thus 21, and the skeleton that shown in formula II. Other substituted groups introduce oxygen atoms either in hydroxyl groups or as a ketone. These are found on carbon atoms 3, 11, 17, 20, and 21.

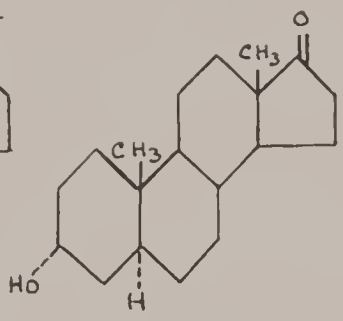
The chemical structure of the compounds belonging to each of the above type of hormone will be discussed briefly as these are considered.

## ANDROGENS

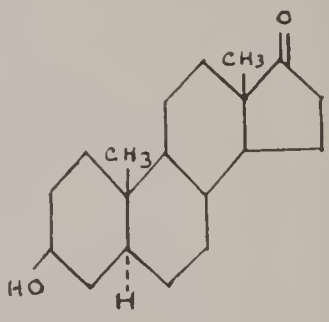
These all contain methyl groups on both carbon atoms 10 and 13, but not the side chain at 17, so that they are $C_{19}$ compounds. Although testosterone is the most active of the androgens, the substances which are determined by the chemical method given below, are metabolites excreted in the urine. These include androsterone, isoandrosterone, dehydroisoandrosterone, and etiocholanolone, all of which are seen to have a ketone group at carbon atom 17, and so are known as 17-ketosteroids.



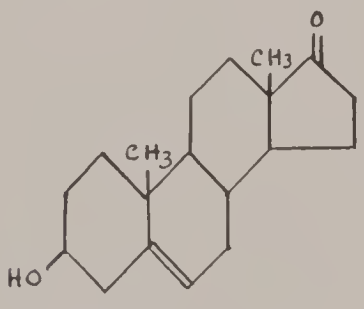

Testosterone



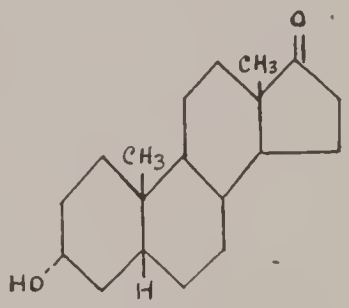

Androsterone




Isoandrosterone




Dehydroisoandrosterone




Etiocholanolone

The isomerism of androsterone and isoandrosterone arises from the hydroxyl group and the hydrogen atom on carbon 3. In one case the hydroxyl group can be imagined to be below the plane of the ring (shown - - - -) and in the other to be above the plane of the ring (shown ——). The two groups of compounds thus possible are designated 3$\alpha$ and 3$\beta$ compounds. Androsterone and etiocholanolone belong to the $\alpha$ group, isoandrosterone and dehydroisoandrosterone to the $\beta$ group. Further stereoisomers arise from the hydrogen atom on carbon 5. This may be above the plane of the ring (shown ——)

as in etiocholanolone, or below (shown - - - -) as in androsterone and isoandrosterone. The latter are sometimes distinguished by the prefix allo-.

It will be noticed that only in dehydroisoandrosterone of the 17-ketosteroids is there a double bond present, between carbon atoms 5 and 6. Otherwise all the carbon atoms have their full complement of hydrogen atoms. Testosterone on the other hand has one double bond between carbon atoms 4 and 5.

### Determination of Neutral 17-Ketosteroids in Urine

Methods for determining 17-ketosteroids in urine are mostly modifications of Callow's original method (Callow *et al.*, 1938) which used coupling of the ketone group at position 17 with *m*-dinitrobenzene in the presence of strong alkali, a reaction introduced by Zimmermann. The resulting colour is compared with that produced from a standard of pure crystalline 17-ketosteroid such as androsterone. Before carrying out the Zimmermann reaction the urine is boiled with acid. After the acid hydrolysis the ketosteroids are extracted with ether or carbon tetrachloride and the extract washed with alkali to remove phenolic compounds. The solvent is then evaporated off and the colour developed on the residue dissolved in alcohol. Much work has been done on this determination. See, for example, Talbot *et al.*, (1940 (2)); Holtorff and Koch, (1940); Robbie and Gibson (1943); Hain, (1947 (1)); Tompsett, (1949). The subject is also reviewed by Callow (1950), and by Mason and Engstrom (1950). Recently a Medical Research Council Committee on Clinical Endocrinology (1951) has published a method which it is hoped will make the results of different laboratories more strictly comparable. This method is given here.

**Reagents.** 1. Concentrated hydrochloric acid.

2. Sodium hydroxide, 2 N solution.

3. Carbon tetrachloride.

4. Absolute alcohol (aldehyde free). The quality of the alcohol used is most important in obtaining low reagent blanks. If unsatisfactory, alcohol can be purified as follows. Allow absolute alcohol to stand in contact with *m*-phenylenediamine hydrochloride (about 4 grams per litre) in the dark for about a week, shaking occasionally. Distil off the alcohol in an all-glass apparatus, discarding the first and last portions. Keep this purified alcohol in the dark. Use whenever alcohol is required in this determination. British Drug Houses' Analar alcohol is satisfactory without further purification.

5. Two per cent. *m*-dinitrobenzene in alcohol. Use specially purified *m*-dinitrobenzene dissolved in aldehyde-free alcohol. Obtain B.D.H. *m*-dinitrobenzene (specially purified) and purify further by the method described by Callow *et al.*, (1938). Dissolve 20 grams in 750 ml. of 95 per cent. alcohol warmed to 40° C. and add 100 ml. of 2 N sodium hydroxide. After five minutes cool the solution and add 2,500 ml. of water. Collect the precipitated *m*-dinitrobenzene on a Buchner funnel, wash very thoroughly with large amounts of water, suck dry, and recrystallize twice in succession from 120 ml. and 80 ml.

of absolute alcohol. The material should be obtained as almost colourless needles with melting point 90·5° to 91° C. On mixing a 1 per cent. alcoholic solution with an equal volume of 2 N sodium hydroxide no colour should result after standing for one hour. Keep the 2 per cent. solution (w/v) in alcohol prepared from this specially purified material, in a brown glass-stoppered bottle in the dark. It will keep for one to two weeks.

6. Potassium hydroxide (carbonate-free), 2·5 N solution in absolute alcohol. Dissolve 9 grams of potassium hydroxide, with shaking, in 50 ml. of absolute alcohol and filter through a hardened filter paper. Titrate against standard acid using methyl orange, and adjust to between 2·48 and 2·52 N. Keep in a refrigerator and discard if any colour develops. This is best prepared freshly at least once a fortnight.

7. Standard solution of dehydroisoandrosterone or androsterone. Prepare a solution containing 1 mg. per ml. of absolute alcohol.

**Technique.** Collect a twenty-four-hour specimen of urine in a Winchester quart bottle. It is not necessary to add a preservative, if the estimation can be begun soon after completing the collection, but 5 to 10 ml. of concentrated hydrochloric acid can be used if the urine has to be kept. Measure out 100 ml. of urine and bring to the boil in a flask fitted with a reflux condenser, using all-glass apparatus. Then pour 10 ml. of concentrated hydrochloric acid down the condenser and boil for ten minutes. Allow to cool a little and then run 30 ml. of carbon tetrachloride down the condenser. Again boil for ten minutes. Cool, remove the carbon tetrachloride layer, add a further 30 ml. of carbon tetrachloride and reflux for another ten minutes. Again cool, remove the carbon tetrachloride layer and add to the first extract. Wash the combined extracts successively with 20 ml. of water, 20 ml. of 2 N sodium hydroxide, 20 ml. of water, and 20 ml. of water containing a pinch of sodium dithionite $Na_2S_2O_4$. Shake with a little anhydrous sodium sulphate, filter through glass wool, washing through with more carbon tetrachloride. Evaporate off the carbon tetrachloride on a water bath. Use a water-pump vacuum to remove the last traces. Dissolve the dry residue in the absolute alcohol. For normal urines 2 or 4 ml. is a convenient volume to use, but more or less should be used if abnormal amounts of ketosteroids are present. This solution keeps well when well stoppered.

For the colour development set up the following tubes :

1. The unknown :
   0·2 ml. of the alcohol solution of the residue from the urine extract.
   0·2 ml. of 2 per cent. *m*-dinitrobenzene in alcohol.
   0·2 ml. of 2·5 N alcoholic potassium hydroxide.
2. Standard :
   0·2 ml. of a solution of dehydroisoandrosterone or androsterone in alcohol containing 0·1 mg. (Use the above standard diluted 1 in 2 with alcohol.)
   0·2 ml. of 2 per cent. *m*-dinitrobenzene in alcohol.

0·2 ml. of 2·5 N alcoholic potassium hydroxide.

3. Reagent blank :
   0·2 ml. of absolute alcohol.
   0·2 ml. of 2 per cent. *m*-dinitrobenzene in alcohol.
   0·2 ml. of 2·5 N alcoholic potassium hydroxide.

Stopper the tubes and place in a water bath at 25 ± 1° C. in the dark, for one hour. Then remove from the bath, add 10 ml. of 70 per cent. alcohol, mix, and read in the photoelectric colorimeter using two filters, first a green one, transmission at 520 millimicrons, and then a violet, transmission at 430 millimicrons.

Readings are made using two filters so that a correction can be made for interfering chromogens. Substances which give a general absorption when treated with Zimmermann reagent are present in urine extracts obtained as described above. The observed reading for the green filter can be corrected for these by means of the formula:

$$\text{Corrected green extinction value} = \frac{\text{Observed green extinction value} - 0{\cdot}6 \times \text{Observed violet extinction value}}{0{\cdot}73}.$$

This formula was suggested by Fraser *et al.* (1941) and Talbot *et al.* (1942). See also Engstrom and Mason (1943) and Butt *et al.* (1951). Engstrom and Mason describe how the correction factors can be derived using the separation into ketonic and non-ketonic fractions by means of Girard's reagent T as carried out by Talbot *et al.* (1940 (3) ). Interfering chromogens are in the non-ketonic fraction. Variations in the correction formula have been found, so that rather more accurate results may be obtained if this is determined for a particular set of conditions. This applies more especially to low values of 17-ketosteroids.

**Calculation.** Mg. 17-ketosteroids per twenty-four-hour specimen

$$= \frac{\text{Corrected reading of unknown}}{\text{Reading of standard}} \times \text{Mg. 17-ketosteroid in the standard used} \times \frac{\text{Volume of alcohol used to dissolve the extract}}{0{\cdot}2} \times \frac{\text{Volume of 24-hour specimen}}{100}$$

So if the extract was dissolved in 2 ml. of alcohol and the standard used contained 0·1 mg., then

$$\text{Mg. 17-ketosteroids per twenty-four-hour specimen} = \frac{\text{Corrected reading of unknown}}{\text{Reading of standard}} \times \frac{\text{Volume of 24-hour specimen}}{100}$$

Construct a standard curve using the standard solution (1 ml. = 1 mg.) given above and setting up tubes as follows :

| | | | | | |
|---|---|---|---|---|---|
| Mg. 17-ketosteroids per 100 ml. urine | 0 | 0·5 | 1·0 | 1·5 | 2·0 |
| Ml. standard solution of a pure 17-ketosteroid (0·2 ml. = 0·2 mg.) . . | 0 | 0·05 | 0·10 | 0·15 | 0·20 |
| Ml. absolute alcohol . . . | 0·20 | 0·15 | 0·10 | 0·05 | 0 |
| Treat as described above. | | | | | |

These figures apply if 2 ml. of alcohol was used to dissolve the extract from 100 ml. of urine.

Take care to see that the straight part of this curve is used, diluting the alcohol solution of the urine extract if necessary. In most cases the amount of colour appears to be proportional to concentration only up to 0·1 mg. of 17-ketosteroid in the actual test sample.

**Notes.** Mason and Engstrom (1950) suggest that dehydroisoandrosterone is the most satisfactory of the 17-ketosteroids to use as standard, and androsterone the least satisfactory. They also state that the amount of colour obtained from the same amount of standard changes with the age of the reagents so that a standard should be put up with each set of determinations if possible. The author has also found this to be the case and would emphasize the desirability of doing a standard with each batch. When the reading of the blank increases the difference between the readings of the standard and the blank decreases, but reproducible results for unknowns are obtained on applying the above correction formula. Wilson and Carter (1947) found that the potassium hydroxide could be stabilized by adding ascorbic acid. See also addendum 1, p. 458.

Hormone metabolites other than 17-ketosteroids may also give some colour with the Zimmermann reaction. Thus the small increases reported in pregnancy are probably due to 20-ketosteroids resulting from the increased production of progesterone. It also appears possible that the increase in 17-ketosteroids as measured by the Zimmermann reaction found in some adrenal diseases is also in part due to the excretion of other metabolites.

### Reaction with Antimony Trichloride (Pincus, 1943)

Pincus showed that antimony trichloride gives a blue colour with the above 17-ketosteroids with the exception of dehydroisoandrosterone. Accordingly the difference between the 17-ketosteroids determined by the Zimmermann reaction and by the Pincus reaction (see Salter *et al.*, 1946) should give a measure of dehydroisoandrosterone. The significance of this is discussed later.

## Application of Chromatographic Methods to the Separation of 17-Ketosteroids

Chromatographic separation of urinary 17-ketosteroids has been carried out by Dingemanse *et al.*, (1946, 1952) and has been used by Robinson and Goulden (1949), and Pond (1951). Zaffaroni *et al.*, (1949) have used paper chromatography.

### Zimmermann Reaction on Serum

Zimmermann has applied his reaction to the determination of 17-ketosteroids in serum. For details see Callow (1950).

INTERPRETATION

The 17-ketosteroids determined by this method are derived from the androgenic hormones of the testis and adrenal cortex. It is considered that androsterone and etiocholanolone arise from the testis, all four from the adrenal cortex.

In males the amount excreted is thus a measure of the combined testicular and adrenal cortical secretion of androgens, in the female of those from the adrenal cortex only. Since both adrenal cortex and testis are under the hormonal control of the anterior pituitary, changes in urinary 17-ketosteroid excretion are found in diseases involving any of these organs—anterior pituitary, adrenal cortex and testis.

The normal values can be summarized as follows :

| | | |
|---|---|---|
| Adult males | 9 to 24 mg. per twenty-four hours. | Average 15 mg. |
| Adult female | 5 to 17 mg. per twenty-four hours. | Average 10 mg. |

The difference is due to testicular secretion in males. On an average about twice as much is derived from the adrenal cortex as from the testis.

These figures are for young adults aged about twenty-five years, at which 17-ketosteroid excretion is at a maximum. A slow fall follows thereafter with advancing years. Thus Hamburger (1948) found at fifty years an average of 10 mg. (range 5–18 mg.) for men and of 6 mg. (range 3–9 mg.) in women.

Before puberty the excretion is the same in both sexes. For children the following figures have been given (Talbot *et al.*, 1940 (1) ; Hain, 1947 (2) ) :

| | |
|---|---|
| Up to 7 years . . . . | 0·5 to 2·5 mg. daily. |
| 8 to 12 years . . . . | 2 to 5 mg. daily. |
| 12 to 15 years . . . . | 5 to 11 mg. daily. |

However, no correction was made for interfering chromogens. Wood and Gray (1949–50) have recently made a study of 17-ketosteroid excretion in children and have applied such corrections. Their results are summarized as follows :

| Age | Sex | No. of cases | Total neutral 17-ketosteroids | | | |
|---|---|---|---|---|---|---|
| | | | Uncorrected | | Corrected | |
| | | | Range | Mean | Range | Mean |
| 2–6 | F | 8 | 1·06–2·8 | 1·65 | 0·29–1·02 | 0·67 |
| | M | 13 | 0·84–3·0 | | 0·32–1·24 | |
| 6–9 | F | 10 | 1·22–4·74 | 2·38 | 0·26–1·55 | 0·89 |
| | M | 13 | 1·26–3·95 | | 0·34–1·50 | |
| 9–12 | F | 12 | 0·50–4·10 | 2·23 | 0·23–2·30 | 1·03 |
| | M | 7 | 1·04–4·00 | | 0·66–1·60 | |

*Decreased excretion of* 17-*ketosteroids* is found when there is hypoactivity of adrenal cortex, pituitary or testis.

1. **Hypopituitarism** (Simmonds' disease). In this condition diminished secretion of both the adrenocorticotrophic and gonadotrophic hormones of the anterior pituitary causes a reduction in the output of androgens by both adrenal cortex and testis. Figures for 17-ketosteroid excretion range from low normal values to almost complete absence depending on the extent of the pituitary deficiency. The estimation may be useful in differentiating the condition from anorexia nervosa, in which normal or only slightly reduced values are found.

2. **Hypogonadism.** In males this may either be due to disease of the testes or be secondary to a reduction in the secretion of the gonadotrophic hormone of the pituitary due to hypopituitarism. It is only in these latter cases that really low figures for 17-ketosteroid excretion are found. In primary hypogonadism adrenal cortical steroids are little affected so that there is only a reduction in testicular hormones. Since only about a third of the urinary 17-ketosteroids are derived from these, findings are low in the normal range or are slightly reduced.

In females, primary ovarian failure has no effect on 17-ketosteroid excretion but this is low or absent if the hypogonadism is due to hypopituitarism. Some degree of hypogonadism may occur associated with increased adrenocortical activity in which condition there is increased excretion of 17-ketosteroids.

In hypogonadism due to primary ovarian or testicular failure there is increased excretion of gonadotrophins by the pituitary, whereas in secondary hypogonadism due to hypopituitarism, gonadotrophins are low or absent. Chemical methods for assaying gonadotrophins are not available. Animal methods must be used (see Venning (1950), and Hamburger (1950) ).

3. **Hypoadrenalism** (Addison's disease). In women, 17-ketosteroids are very low or absent, but in men, because the testes usually remain active, there is still an excretion of from 1 to 6 mg. even in the most severe cases.

4. Excretion of 17-ketosteroids is also often rather low in **myxœdema** and tends to be on the low side in many severe illnesses.

*Increased excretion of* 17-*ketosteroids* is most characteristic of increased activity of the adrenal cortex, but high outputs have also been reported in certain types of tumour of the testis.

1. **Hyperadrenalism** may be due to hyperplasia or to tumours of the adrenal cortex. It is in the second group of cases that the highest values for urinary 17-ketosteroids have been found, often up to 300 mg. per twenty-four hours, and occasionally even higher. In particular, cases of prepubertal virilism give very high figures. There is an appreciable amount of overlapping between tumour and non-tumour cases, so that a means of distinguishing these is desirable, and one has been suggested based on the type of 17-ketosteroids excreted in the urine in patients with an adrenal tumour. Callow

(1936, 1939) showed that relatively large amounts of dehydroisoandrosterone are excreted in such cases. Talbot *et al.*, (1940 (4)) showed that the 17-ketosteroids can be separated into α- and β-17-ketosteroids (see p. 434) by precipitation with digitonin since the α are not precipitated while the β are. Butt, Henley and Morris (1948), Frame (see Venning, 1950), and Haslam and Klyne (1952), have also published methods for separating these by means of digitonin. The α-17-ketosteroids (androsterone and etiocholanolone) form 85 to 95 per cent. of the total in normal persons, according to Butt *et al.*, from 81 to 99 per cent. according to Haslam and Klyne. The marked increase in dehydroisoandrosterone which occurs in patients with adrenal tumours causes a considerable increase in the percentage of β-17-ketosteroids, and this was used diagnostically by Talbot. Subsequent work has further confirmed this excretion of relatively large amounts of dehydroisoandrosterone in adrenocortical tumours, particularly in females. The author has found the following method of fractionation satisfactory.

**Fractionation into α and β Fractions** (Haslam and Klyne, 1952; Butt, Henley and Morris, 1948)

After being treated with an alcoholic solution of digitonin, β-ketosteroids are precipitated as a digitonide complex by ether, while the α-ketosteroids remain free in the ether. The digitonin complex is decomposed by treating with dry pyridine, so that on adding ether the digitonin from the complex is precipitated and the β-ketosteroids remain in solution. The two fractions are then assayed separately by the Zimmermann reaction.

**Reagents.** 1. Digitonin—a 1 per cent. w/v solution in warm 90 per cent. v/v ethyl alcohol. Keep at 37° C. in a tightly stoppered container.

2. Ether, peroxide-free.

3. Pyridine. Dry over barium oxide and redistil.

4. Absolute ethyl alcohol. The specially pure alcohol used in the 17-ketosteroid determination (p. 435) must be employed throughout.

5. Sulphuric acid, 2 N.

**Technique.** Take an amount of alcohol extract prepared as described above, which contains 1·0 to 1·5 mg. of ketosteroid (and not more than 0·5 mg. of the β-fraction). Evaporate in a graduated centrifuge tube. Add 0·75 ml. of the warm digitonin solution. Heat quickly to boiling over a microflame. Stopper and leave in the refrigerator overnight. Add 10 ml. of ether with stirring in small amounts as follows. First add 3 ml. in two 1·5 ml. portions at an interval of a few minutes. Then allow the tube to stand for about half an hour, stirring occasionally with a fine glass rod. At the end of this time add a further 4 to 5 ml. of ether. After a further period of standing, making a total of forty-five minutes standing, add the final 2 to 3 ml. of ether, using this to wash the glass rod and the inside of the tube. Centrifuge and decant the clear supernatant fluid into a small separating funnel. Wash the precipitate three times with 5 ml. of ether, stirring, centrifuging, and decanting the supernatant fluid

each time. Standing is not neeessary if the initial floeeulation was satisfaetory. Wash the eombined supernatant fluids in the separating funnel three times with 5 ml. of water, transfer to a small flask and remove the ether under redueed pressure at room temperature. Dry for an hour over ealeium chloride. This gives the $\alpha$ fraetion.

Dry the preeipitate in the eentrifuge tube in air and for a further twenty minutes in a desieeator over ealeium chloride with eontinuous evaeuation. Then dissolve in 0·25 ml. of dry pyridine, warming in a water bath at 60° to 70° C. for three minutes. Cool in running water, add 5 ml. of ether as above in small amounts with stirring, allowing the tube to stand for about fifteen minutes before adding the last 2 ml. Centrifuge, deeant the elear supernatant fluid into a separating funnel. At onee make a seeond pyridine-ether extraetion. Wash the preeipitate twiee with ether. Wash the eombined extraets and washings twiee with 5 ml. of 2 N sulphurie aeid and three times with 5 ml. of water. Transfer to a small flask, remove the ether under redueed pressure and dry the residue as above. This gives the $\beta$ fraetion.

Take up the residues of the $\alpha$ and $\beta$ fraetions in 2 ml. and 0·5 ml. of aleohol respeetively and use these for earrying out the Zimmermann reaetion as deseribed for 17-ketosteroids earlier (p. 436).

### Patterson's Test for Excess Dehydroisoandrosterone

Patterson (1947) has reported a simple eolour test for revealing the presenee of dehydroisoandrosterone in these eases. He found that this substanee gives an intensely violet blue eolour with some green fluoreseenee when it is treated with sulphuric aeid. He found that the reaetion eould be made sensitive to less than 0·1 mg., and showed that it could be used to deteet an exeess of dehydroisoandrosterone in the extraets of 17-ketosteroids obtained as deseribed above.

For extraetion of 17-ketosteroids, Patterson used 25 ml. of urine and hydrolyzed by refluxing with 0·75 ml. of eoneentrated sulphurie aeid for thirty minutes. After extraeting with ether and washing with alkali and water, he dissolved the residue obtained by evaporating off the ether, in 1 ml. of absolute aleohol. For the test for dehydroisoandrosterone he took 0·2 ml. of this, eorresponding to 5 ml. of the urine. If the method (p. 436) is used take a volume of aleohol extraet eorresponding to this volume of urine.

Transfer the appropriate amount of this aleohol solution to a dry test tube and evaporate eompletely to dryness in a vaeuum desieeator. Add 1 ml. of eoneentrated sulphurie aeid and shake the tube until the extract is eompletely dissolved. Plaee in a water bath at 25° C. for twenty minutes. Then immerse in eold water, add 1 ml. of distilled water, drop by drop down the side, shaking eontinuously. Heat the diluted solution in a bath of boiling water for one minute during which time a blue eolour develops if dehydroisoandrosterone is present in significant amounts. The eolour is very stable and is not altered appreciably if the final heating is done for several minutes. Urines from eases other than adrenal tumours give various shades of brown.

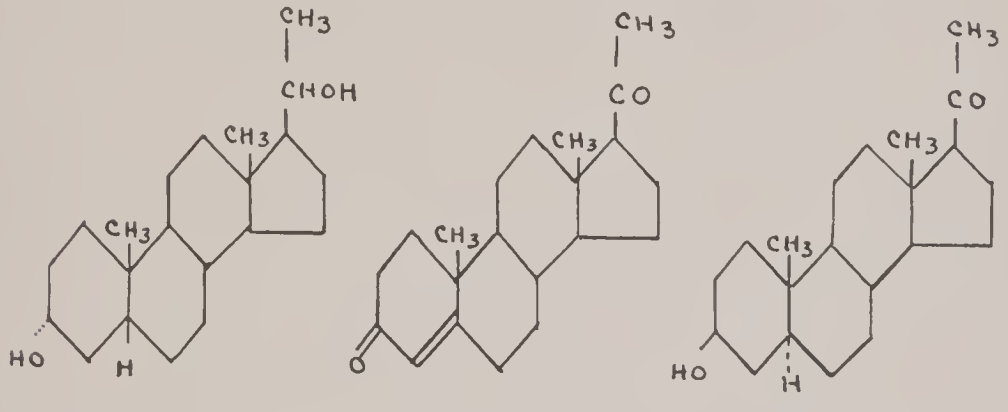
Patterson reports findings from three cases of adrenal tumours, all in females, which gave intense colours, the twenty-four hour output of 17-ketosteroids being 215, 335, and 1,980 mg. Completely negative results were obtained from six cases of prepubertal virilism with a twenty-four hour output from 24 to 81 mg. Eight cases of secondary virilism with 21 to 30 mg. daily excretion gave completely negative results. Subsequent work supports the value of this test though examination of a greater number of cases of adrenal tumour with smaller outputs of 17-ketosteroids than those given above is desirable.

2. **Tumours of the Testis.** Greatly increased excretion has been found associated with interstitial cell tumours but in other testicular tumours, normal or reduced amounts are excreted. Following administration of testosterone propionate about 50 per cent. of it appears in the urine as 17-ketosteroids.

**Acromegaly** does not lead to increase in excretion of 17-ketosteroids and the high values found in Cushing's syndrome when due to tumour of the adrenal cortex do not seem to be found when the condition is due to primary pituitary disease (Fraser *et al.*, 1941).

## HORMONES OF THE CORPUS LUTEUM. PREGNANEDIOL

Pregnanediol is derived from progesterone, the hormone secreted by the corpus luteum and the placenta. These two substances are $C_{21}$ compounds since they have the same two methyl groups as the 17-ketosteroids and also a two carbon atom side chain on carbon atom 17.




Pregnanediol Progesterone Pregnanolone

Pregnanediol is excreted in the urine as the glucuronide. Also present is some pregnanolone, also as glucuronide. This contains two hydrogen atoms less on carbon atom 20 and also differs from pregnanediol in belonging to the other isomeric group arising from the position of the hydrogen on carbon atom 5, that is, it belongs to the allo- series.

### Determination of Pregnanediol in Urine

The first method used was that of Venning (1937, 1938), later

modified (Venning, 1950). The pregnanediol glucuronide is extracted from the urine, carefully purified and weighed. Although widely used this method is rather laborious and is only satisfactory for urines containing more than 10 mg. of pregnanediol. Furthermore, the urine must be carefully preserved to prevent hydrolysis of the glucuronide. Astwood and Jones (1941) hydrolyzed the glucuronide by boiling the urine with acid, extracted the free pregnanediol with toluene, and purified and weighed this. Talbot *et al.*, (1941), developed a colorimetric method in which they determined the free pregnanediol extracted by the method of Astwood and Jones, by means of the yellow colour which it gives with concentrated sulphuric acid. This considerably increased the sensitivity of the method. Gutermann (1944, 1945) developed simpler methods from this. A careful study of the colorimetric method was made by Somerville, Gough and Marrian (1948), who published a method which is probably the most accurate available at present. Somerville, Marrian and Kellar (1948) then developed a modification of this method which could be carried out on 100 ml. of urine and so only requires the collection of a single morning specimen. This last method has been further studied and slightly modified by Henderson *et al.*, (1949). Tompsett (1950) has published a modified gravimetric method which weighs free pregnanediol. Finally, it may be noted that Mack and Parks (1947, 1949) extracted the pregnanediol from the whole of an overnight specimen of urine and roughly estimated this by assessing the amount of turbidity produced when it was precipitated from acetone by dilute sodium hydroxide.

### Gravimetric Estimation as Sodium Pregnanediol Glucuronide

Method of Venning (1937, 1938, 1950 ; see also Haslewood, 1950)

This method is the modification of her earlier methods given by Venning (1950).

**Reagents.** 1. Butyl alcohol. Bisset *et al.*, (1948) recommend purification to remove substances which destroy the glucuronide when the butyl alcohol extract is evaporated *in vacuo*. Distil the commercial product at 112° to 118° C. over potassium hydroxide (5 grams per litre). Then redistil at about 70° C., 20 mm. mercury, with an air leak, and with *p*-aminophenol (1 gram per litre) and 5 N sodium hydroxide (1 ml. per litre) added. Finally, redistil at 60° to 70° C., 20 mm. mercury.

2. N/10 Sodium hydroxide.

3. Acetone.

4. Ethyl alcohol, 94 per cent. (v/v).

**Technique.** Collect a twenty-four hour specimen of urine. Since the sodium pregnanediol glucuronide breaks down to give free pregnanediol on standing, careful preservation of the urine is essential. Venning (1938) found toluene and chloroform unsatisfactory, but 0·5 per cent. sodium cyanide kept the urine for four days at a temperature below 20° C. Later Venning (1950) used 5 to 6 drops of tricresol per litre, or 100 ml. butyl alcohol and kept in the

refrigerator until used. Bisset *et al.* (1948) used 3 grams of a mixture of 2 parts thymol to 1 part of salicylic acid, finely powdered.

Use a volume of urine which is thought to contain 10 to 20 mg. of conjugated pregnanediol, that is, about 6 to 12 mg. of free pregnanediol. To get such an amount, it may be necessary to use up to a forty-eight hour specimen when examining urines from an ordinary menstrual cycle or in early pregnancy, but in later pregnancy a litre or less will be sufficient.

Extract the urine three times with normal butyl alcohol using 200, 100, and 100 ml. per litre of urine. Shake *gently* twenty to twenty-five times at each extraction and stand about fifteen minutes for separation of the layers. Avoid vigorous shaking since this extracts more impurities and so gives a less pure final precipitate. Shake the combined extracts vigorously in a separating funnel and stand up to an hour to obtain a clear butyl alcohol layer. Run off the lower urine layer which then separates, and discard. Evaporate the clear butyl alcohol layer almost to dryness under reduced pressure, dissolve in 60 ml. of N/10 sodium hydroxide, and extract successively with 30, 20, and 10 ml. of butyl alcohol. Wash the combined extracts twice with 5 ml. of distilled water. Evaporate to dryness under reduced pressure. Dissolve the residue in exactly 5 ml. of water and transfer to a conical flask washing out with small volumes of water. Bring the volume up to 100 ml. by adding chilled acetone. Then keep at — 10° C. for one hour, centrifuge the mixture for ten minutes and remove the supernatant fluid without disturbing the deposit. This precipitate is next dissolved in water, the volume used depending on the weight of deposit. Use 2 ml. of water if the weight is less than 5 mg., 3 ml. if between 5 and 10 mg., and 5 ml. if over 10 mg. First add this water to the conical flask already used, dissolve any precipitate still remaining in this, and then run the liquid into the centrifuge tube containing the precipitate. Dissolve with gentle warming and then, using suction and a small paper, filter into the conical flask already used. Wash the tube and the paper with small volumes of acetone. Bring the volume to 100 ml. with chilled acetone and place in the refrigerator for an hour. Again centrifuge and remove the supernatant fluid without disturbing the deposit. Dissolve the precipitate in 10 ml. of hot 94 per cent. ethyl alcohol and filter into a 30 ml. tared beaker. Wash out the conical flask and centrifuge tube with small volumes of the hot alcohol and filter this also. Evaporate to dryness on a water bath under a stream of air. Dry the residue *in vacuo* over phosphorus pentoxide. Weigh the residue. This melts at 273° C. with decomposition and evolution of gas.

**Calculation.** There is some loss of glucuronide with each acetone precipitation. This is proportional to the amount of precipitate and to the volume of water used for the second precipitation. The table gives the approximate recovery under the varying conditions that might be used. The amount of sodium pregnanediol glucuronide obtained is corrected for this loss and multiplied by the factor 0·618 to convert it into free pregnanediol. Although the precipitate

is not entirely pregnanediol but contains some pregnanolone this does not make any significant change in the factor.

TABLE XXIX

*Recovery of Sodium Pregnanediol Glucuronide*

(*From "Methods in Medical Research," Vol. 2. Edited by J. H. Comroe. Year Book Publishers, Chicago*)

| | Water used in Second Precipitation | | |
|---|---|---|---|
| | 2 ml. | 3 ml. | 5 ml. |
| Mg. precipitate | % recovery | % recovery | % recovery |
| 3–4 | 62 | 60 | — |
| 5–8 | — | 70 | 65 |
| 9–12 | — | — | 71 |
| 13–18 | — | — | 80 |
| 19–25 | — | — | 85 |

Hence Mg. Pregnanediol excreted daily

$$= \frac{\text{Weight of precipitate} \times \text{volume of twenty-four hour specimen} \times 0{\cdot}618 \times \text{recovery factor}}{\text{Volume of urine extracted}}$$

### Method of Sommerville, Gough and Marrian (1948)

**Reagents.** 1. Toluene. Redistilled "sulphur free."
2. Concentrated hydrochloric acid. (Analytical reagent).
3. Normal sodium hydroxide.
4. Ethyl alcohol.
5. N/10 sodium hydroxide.
6. Hyflo-Super Cel. From Johns-Manville Co. Ltd.
7. Norit charcoal.
8. Concentrated sulphuric acid.
9. Pure pregnanediol.

**Technique.** Collect a twenty-four-hour specimen of urine using 5 ml. of toluene as preservative. Make up to 2,500 ml. and take duplicate 500 ml. samples. Place each sample in a litre flask, add 100 ml. toluene and bring to the boil under a reflux condenser, using all-glass apparatus (Quickfit and Quartz). Run 50 ml. of concentrated hydrochloric acid down the condenser into the boiling mixture, and continue boiling for exactly ten minutes. Cool the flask rapidly in cold water and transfer the mixture to a 750 ml. separating funnel. Shake and allow the urine layer to separate. Run this off into the original flask, then filter the layer of toluene and emulsion through a Whatman No. 1 filter paper on a Buchner filter funnel. Return the urine layer to the separating funnel and extract twice more with 100 ml. portions of toluene, each time filtering the toluene and emulsion layer through the same filter funnel. Transfer the combined filtrates to a clean separating funnel, run off the small urine layer that separates, wash the toluene extract twice with 100 ml. of normal sodium hydroxide and twice with 100 ml. of water. Run the washed toluene extract into a 500 ml. round-

bottomed flask and evaporate nearly to dryness on an electric hot plate and then completely to dryness under reduced pressure on a boiling water bath.

Transfer the dry residue quantitatively with warm ethyl alcohol to a 20 ml. centrifuge tube and evaporate to dryness in a water bath whilst passing a stream of air. Add 4 ml. ethyl alcohol to the residue and place the tube in a beaker of water at 75° C. Stir with a glass rod for a minute to dissolve completely, then add 16 ml. of N/10 sodium hydroxide drop by drop from a burette taking three minutes and stirring continuously and using the last ml. to wash the stirring rod. After a further minute at 75° transfer the beaker of water and tube to a 37° C. incubator and leave overnight. Add about 8 to 10 mg. of Hyflo-Super Cel stirring with a glass rod, which is then washed with 1 ml. of a 1 : 4 (v/v) ethyl alcohol-water mixture to avoid loss of material on the rod. Centrifuge the tube for an hour at 1,500 r.p.m. Carefully suck off the supernatant fluid from the precipitate using a fine glass tube.

Carry out two further precipitations in the same way, using water instead of sodium hydroxide solution and incubating for only two hours. It is not necessary to use any of the Hyflo-Super Cel before centrifuging after these two precipitations.

Add 5 ml. of ethyl alcohol to the final precipitate and dissolve by warming with stirring at about 75° C. Add 1 to 2 mg. of Norit and warm for a further two minutes. Filter through a small Whatman No. 1 filter paper into a test tube 1 inch in diameter, washing the centrifuge tube and filter three times with 2 ml. of warm alcohol. Evaporate filtrate and washings in a water bath using a stream of air and complete the drying by placing in a vacuum desiccator over calcium chloride for several hours.

Carry out the colour reaction with not more than about 0·5 mg. of the final product. Should there appear, on inspection, to be more than this amount, dissolve in ethyl alcohol and take a suitable aliquot. Now add to the dry pregnanediol, 10 ml. of concentrated sulphuric acid from a burette and leave in a water bath at 25° C. for twenty minutes, shaking occasionally. Measure the amount of yellow colour produced, in the photoelectric colorimeter, using a violet filter or transmission at 420 millimicrons. Prepare a calibration curve using amounts of pure pregnanediol varying from 0·1 to 0·5 mg. Check this curve frequently.

It is claimed that this method is satisfactory for amounts of pregnanediol of more than 2 mg. per twenty-four hour specimen. False high results can be obtained from urines containing much increased quantities of neutral 17-ketosteroids.

### Rapid Method of Henderson, Maclagan, Wheatley and Wilkinson (1949)

This is a modification of the more rapid method adapted from the above by Sommerville, Marrian and Keller (1948). The method is stated to detect amounts down to 0·3 mg. pregnanediol per 100 ml. urine.

**Reagents.** 1. Toluene. Redistilled, sulphur free.
2. Concentrated hydrochloric acid. Analytical reagent grade.
3. Sodium hydroxide, 20 per cent. solution.
4. N/10 sodium hydroxide.
5. Sodium hydroxide, 2 per cent. solution in methyl alcohol.
6. Concentrated sulphuric acid. Analytical reagent grade.
7. Petroleum ether, b. p. 40° to 60° C.
8. Acetone.
9. Solution of pregnanediol. One mg. per ml. of alcohol is convenient. Allopregnanediol can be obtained from Organon Ltd.

**Technique.** Measure 100 ml. of urine, 10 ml. of concentrated hydrochloric acid and 50 ml. of toluene into a flask fitted with a condenser with a ground-glass joint and reflux for fifteen minutes. Cool to room temperature and transfer to a separating funnel. Run off the urine layer and wash the toluene extract, first with water, then once with 20 per cent. sodium hydroxide, twice with N/10 sodium hydroxide and lastly with water. Boil the extract in a 100 ml. beaker on an electric hot plate until all traces of water have been removed. Cool a little and add 10 ml. 2 per cent. sodium hydroxide in methyl alcohol (freshly prepared every two to three days). Evaporate down to about 15 ml. on a hot plate. Cool and filter through a sintered glass funnel (porosity 3). The filtrate should have a green fluorescence and be free from any orange or brown tint. Wash the precipitate several times with small amounts of toluene and evaporate filtrate and washings to dryness under reduced pressure. Dissolve in 2·5 ml. of hot acetone, add 47·5 ml. of cold N/10 sodium hydroxide and bring to the boil. Stand at room temperature for one hour. A visible precipitate forms if an appreciable amount of pregnanediol is present. Collect the precipitate on a sintered glass funnel, wash several times with water and then with about 5 ml. of petrol ether. To dissolve the precipitate, pour about 10 ml. of hot acetone through the funnel in several portions, collecting the acetone in a test tube. Carefully evaporate off the acetone in a 50 ml. beaker, and dissolve the residue, when cold, in 10 ml. of concentrated sulphuric acid, and read the colour, using a violet filter as before. Prepare a standard curve using amounts of pregnanediol up to 0·5 mg. per 10 ml. of sulphuric acid. Use the straight line part only of this curve. Carry out a blank on each batch of reagents using normal male urine. Subtract this reading, which should be less than 0·1 mg., from the result in every case.

Clean the glass filters in chromic acid between estimations. Purify the toluene if necessary by washing with methanolic sodium hydroxide and fractional distillation. Do not allow the toluene to come into contact with rubber.

### Isolation of Pregnanediol from Pregnancy Urine (Henderson *et al.*, 1949–50)

Reflux 2·5 litres of pregnancy urine with 250 ml. of concentrated hydrochloric acid for fifteen mimutes. Cool to room temperature and shake with 2 grams of Hyflo-Super Cel and pass through a 3 × 1

inch column of activated alumina (B.D.H.). Wash the column with 100 ml. of water and elute with 250 ml. of hot acetone. Shake the dark brown eluate with 7 grams of calcium oxide. This removes some of the water and some of the colour. Filter and evaporate off the solvent, and extract the residue several times with hot acetone and filter the extract. Evaporate off the acetone and dissolve the residue in 20 ml. of boiling ethyl alcohol. Add 20 mg. of charcoal and filter. Treat the hot filtrate with 80 ml. of hot water and heat to boiling. Stand for two hours to allow complete precipitation. Collect the precipitate and wash with light petroleum (b. p. 40° to 60° C.). To purify, dissolve in 15 ml. of hot acetone, decolorize with charcoal, treat the hot filtrate with 10 ml. of water heated to 60° C. Henderson *et al.*, obtained a product which, crystallized, had a m. p. 232° to 233° C. Further recrystallization from acetone raised the m. p. to 234° C.

INTERPRETATION

Since pregnanediol is derived from progesterone, the hormone of the corpus luteum and placenta, it is present in the urine in appreciable amounts during the luteal phase of the menstrual cycle and in considerable quantities during pregnancy. The normal menstrual cycle can be divided into two approximately equal parts, the follicular phase from the beginning of bleeding to the rupture of the follicle and discharge of the ovum, and the luteal phase beginning with ovulation and formation of the corpus luteum and continuing until bleeding begins again.

**Values for the Normal Menstrual Cycle.** Until recently the Venning method was used by the majority of workers so that most of the figures available were obtained by this method. Very little pregnanediol is excreted during the follicular stage of the menstrual cycle, but beginning about one to two days after ovulation there is increased output to about 3 to 5 mg. daily (Venning and Browne, (1938); Wilson, Randall and Osterberg (1939); Hamblen, Ashley and Baptist (1939); Hain and Robertson (1939); Stover and Pratt (1939); Cope (1940)). From then, until one to three days before bleeding begins, a total amount of 40 to 50 mg. is excreted. However, this quantity is very variable and a range of 3 to 50 has been given by Wooster (1942). Henderson *et al.* found a concentration up to 1·5 mg. per 100 ml. in the morning urine during this period. Because of the variability and the comparatively small quantities involved, the determination cannot easily be used for ascertaining the time of ovulation. Even for showing whether ovulation has occurred the determination must be made over several days to show the excretion of a significant amount.

**Values for Pregnancy.** In pregnancy during the first three months, before the placenta begins to produce progesterone, there is an excretion corresponding in amount to that during the luteal phase of the menstrual cycle. From that point onwards there is a steep rise to a daily output of 40 to 100 mg. (Venning (1938); Wilson and Randall (1938); Jones *et al.*, (1944)). In the days immediately

preceding labour there is a marked fall in the amount of pregnanediol excreted. Attempts have been made to use the determination.

(*a*) *As a Pregnancy Test.* Gutermann (1944, 1945) first suggested use of the pregnanediol excretion in diagnosing pregnancy. He used a roughly quantitative test and claimed 93 per cent. accuracy, a higher figure than that obtained by almost all other investigators. Thus, Reinhart and Barnes (1946) found 69 per cent. accurate, Merivale (1948) 64 per cent., whilst Swyer (1949), using a modified Gutermann procedure on a morning specimen and a minimum level of 4 mg. pregnanediol per litre of urine for a positive test, obtained 67 per cent. correct results. Using the method of Somerville *et al.*, Swyer achieved an accuracy of 82 per cent. Henderson *et al.*, (1949–50) used morning specimens of urine and their method described previously (p. 447). They conclude tentatively that in early pregnancy up to sixteen weeks the concentration of pregnanediol is no greater than that found in the luteal phase of a normal menstrual cycle. A reliable menstrual history is essential in assessing results. Using a value of over 1·5 mg. per 100 ml. as diagnostic, only 27 per cent. of positive results was obtained. A missed period and the presence of pregnanediol, however, was found in 91 per cent. of cases. The authors state that though not observed by them, similar findings have been reported by other workers in cases of luteal ovarian cyst and in arrhenoblastoma of the ovary. The absence of pregnanediol on one occasion, seen in the remaining 9 per cent. of their cases, did not exclude pregnancy. Later repetition usually gave a positive result. A false negative in pregnancy was not obtained later than ten weeks from the last menstrual period.

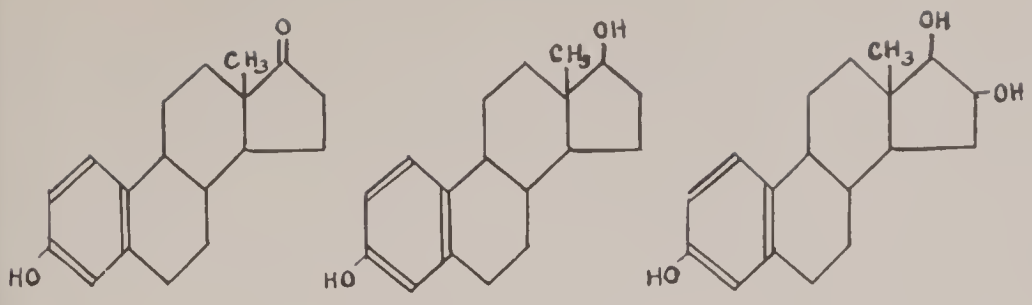
It may be concluded that taken in conjunction with a missed period an accuracy of about 90 per cent. may be achieved. This is less than that obtained with the biological tests in use. Its advantage lies in its speed since a result can be obtained in a few hours, if the Henderson method is used.

(*b*) *Use in Cases of Threatened Abortion.* There is a lower average level of pregnanediol in cases which subsequently abort than those in which pregnancy continues to full term. A fall in pregnanediol excretion often occurs some days before clinical signs appear in cases which abort. Swyer obtained 72 per cent. correct against 93 per cent. by Gutermann (1946). See also Hain (1940), Lyon (1946), and Bender (1948). It has been suggested that the test might give useful prognostic information in patients who have had repeated abortions.

It is still doubtful whether the determination will gain a definite place in ordinary routine use.

## ŒSTROGENS

Pregnancy urine contains three œstrogens, œstrone, œstradiol, and œstriol. The international unit of œstrone is equivalent to 0·1 μg. It is seen that the ring I containing carbon atoms 1 to 6 is phenolic and that there is only one attached methyl group, this being on




œstrone œstradiol œstriol

carbon 18. There is no side chain at 17 so that the œstrogens are $C_{18}$ compounds.

**Chemical Estimation** (Stevenson, M. F., and Marrian, G. F. (1947) )

Most of the work done on the chemical assay of œstrogens has used the Kober method (Kober, 1931). After acid hydrolysis of the glucuronates and sulphates, in which form œstrogens occur in urine, ether extraction is carried out and the ether extract washed with bicarbonate to remove acidic substances. A red colour is then developed with phenol and sulphuric acid. A review of the work done on this reaction is given by Haslewood (1950).

**Reagents.** 1. Concentrated hydrochloric acid.

2. Ether (peroxide free). Shake analar ether with a small amount of 1 per cent. ferrous sulphate solution freshly prepared, decant off the ether, and distil.

3. Sodium bicarbonate, 5 per cent. solution (w/v).

4. Ethyl alcohol.

5. Benzene.

6. Sulphuric acid, 10 per cent. (v/v).

7. Normal sodium hydroxide.

8. Kober reagent. Use pure sulphuric acid and freshly redistilled phenol (measured at 60° C.) in proportion of 14 to 9. Add the acid slowly to the phenol, exclude all traces of moisture, and prevent the temperature from rising. Allow the pipettes to drain thoroughly. Mix well and keep in a glass-stoppered bottle.

9. Standard solution of pure œstriol, 3 mg. per 100 ml., in ethyl alcohol. 1 ml. = 30 $\mu$g.

**Technique.** Collect a twenty-four hour specimen of urine using toluene as a preservative. Dilute to 2·5 litres. Measure out 100 ml. and bring to the boil under a reflux condenser. Run 15 ml. of concentrated hydrochloric acid down the condenser and boil for thirty minutes, cool, and extract three times with ether, using 100 ml., 50 ml., and 50 ml. Wash the combined extracts three times with 25 ml. portions of 5 per cent. sodium bicarbonate. Extract the washings with 20 ml. of ether and add this to the washed ether extract. Evaporate off the ether. Warm the residue with about 3 ml. of ethyl alcohol and add 100 ml. of benzene. Extract three times with N sodium hydroxide, using 50, 25, and 25 ml. Acidify the combined extracts with 15 ml. concentrated hydrochloric acid and extract three

times with ether, using 100, 50, and 50 ml. Wash the combined extracts twice with 20 ml. of 5 per cent. bicarbonate. Again extract the washings with 20 ml. ether and add to the washed ether extract. Wash the whole of this ether extract three times with 20 ml. water.

Evaporate to dryness. Dissolve the residue in ethyl alcohol. For the colour development take a portion of the alcoholic solution, which contains between 10 and 80 μg. of œstrogen and which is equivalent to not more than 2 per cent. of the twenty-four hour urine. If an amount equivalent to more than 2 per cent. is used, a precipitate may form during the fading procedure used later. Evaporate off the alcohol in a stream of air in test tubes about 2 cm. in diameter, graduated at 8 ml. and 15 ml. Add 3 ml. of the Kober reagent to the dry residue and heat the tube in a boiling water bath for twenty minutes. Cool in an ice-salt freezing mixture, add 3 ml. of water, and mix well. Heat for three minutes in a boiling water bath, cool to room temperature by placing in water and make to 15 ml. with 10 per cent. sulphuric acid (v/v). Read in the colorimeter using a green filter, or transmission at 520 millimicrons. Heat 8 ml. left in the tube, in a boiling water bath for one and a half hours to fade the pink colour due to the œstrogens. Cool and make to 8 ml. with water to replace that lost by evaporation. Again read at 520 millimicrons. Take the difference of these two readings and read the amount of œstrogens present (as œstriol) on a calibration curve prepared by treating known amounts of œstriol in the same way. Stevenson and Marrian found that the fading technique gave more significant corrections the smaller the amount of œstrogens and that for urines containing more than about 15 mg. per twenty-four hours a correction was no longer necessary. They conclude also that little quantitative significance can be placed on results giving less than 2 mg. per twenty-four hours.

**Note.** Œstriol is extracted by sodium carbonate from a benzene solution whereas œstrone and œstradiol are not. Separation into these two fractions can be carried out in this way, and the œstrone and œstradiol then estimated by the Kober reaction as above, the œstrone by the Zimmermann reaction with metadinitrobenzene.

INTERPRETATION

The ovary secretes chiefly œstradiol, the placenta chiefly œstriol, though with appreciable amounts of œstradiol and œstrone. During the normal menstrual cycle œstrogens are secreted during both the follicular and luteal phases since they are produced both by the Graafian follicle and by the corpus luteum. The amount excreted increases as the follicle grows so that it is at a maximum when the follicle ruptures and the ovum is released. During the luteal phase there is first a decrease then an increase when the corpus luteum begins to secrete, and finally, a quick fall in the period just before menstruation begins. In pregnancy during the early stages œstrogens are produced by the corpus luteum in amounts similar to those found in the normal menstrual cycle. These do not exceed about 0·1 mg. daily. After about the eighth to twelfth weeks there is a marked rise and much greater amounts are excreted than are found in non-

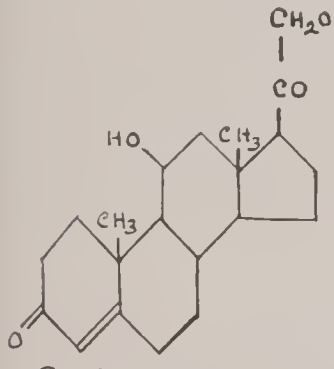
pregnant women. As much as 10 mg. and over may be excreted daily.

The above chemical method can only be used for determining œstrogens in the later stages of pregnancy. Thus Marrian (1946–48) states that it is doubtful whether any of the methods used for correcting for the brown colour produced by non-œstrogenic substances are reliable when less than about 2 mg. of œstrogens are present in a twenty-four hour specimen and concludes that the value of the method when applied to the urine of non-pregnant women is doubtful. Furthermore, it has very little use in disease in non-pregnant women. A small increase has been reported in ovarian granulosa cell tumours and in hyperactivity of the adrenal cortex, but these increases are not sufficiently great to be measured by the chemical method. Increases have also been reported in liver diseases, and are attributed to diminished destruction, inactivation, or excretion by the liver.

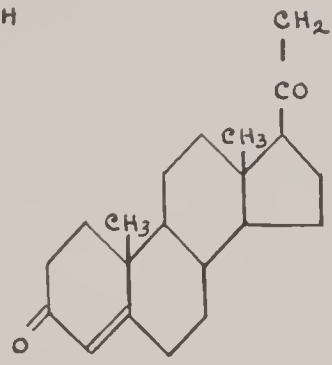
Biological methods are described by Emmens (1951).

## CORTICOSTEROIDS

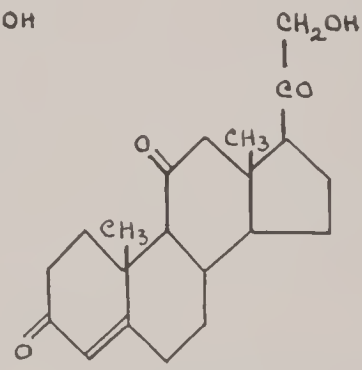
Steroids responsible for the part played by the adrenal cortex in such processes as salt and water regulation and carbohydrate metabolism are known as corticosteroids. They have the full number of 21 carbon atoms shown in formula II (p. 433) since in addition to the two methyl groups on atoms 10 and 13 they have a $-CO.CH_2OH$ side chain at 17. Most have an oxygen atom on carbon atom 11 either as part of a hydroxyl group or forming a ketone group with that carbon atom. For this reason they are also described as 11-oxysteroids. Examples of corticosteroids are



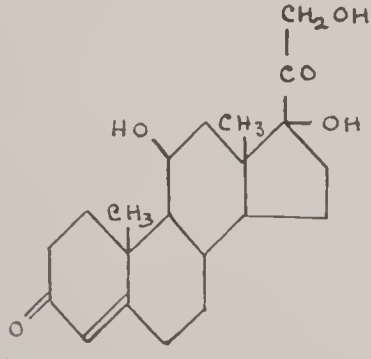

Corticosterone



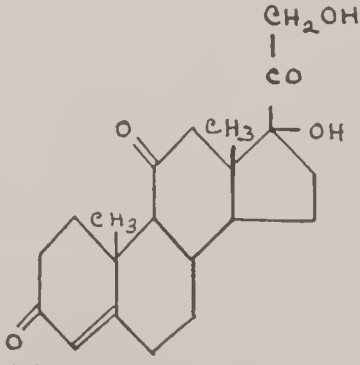

11-desoxycorticosterone




11-dehydrocorticosterone




17-hydroxycorticosterone




11-dehydro-17-hydroxycorticosterone
(Cortisone)

It appears that in steroids responsible for the role of the adrenal cortex in carbohydrate metabolism there must be a ketone group at carbon 3, an oxygen, either hydroxyl or ketone, on carbon 11, and the side chain at 17. For the action on salt metabolism only the groups at 3 and 17 are required. Thus it has been held that desoxycorticosterone does not influence carbohydrate metabolism so that hypoglycæmia still occurs when it is used in the treatment of Addison's disease. However, it has been suggested (Verzer and Wenner, 1948) that under some circumstances desoxycorticosterone can influence carbohydrate metabolism.

Chemical methods used for determining urinary corticosteroids extracted from urines have been of three types.

1. Oxidation of the side chain at 17 by periodic acid to give formaldehyde, a principle used by Corcoran and Page (1948) and by Daughaday *et al.*, (1948). The formaldehyde is distilled over and estimated colorimetrically using the colour given with chromotropic acid. Bassil and Hain (1950) used Conway units for this purpose. Rabinowitch *et al.*, (1951) have published a simplified form of this method.

2. Reduction of phosphomolybdic acid. Molybdenum blue is formed and measured colorimetrically. See Heard and Sobel (1946), and Heard, Sobel, and Venning (1946), and Sprechler (1950).

3. Reduction of alkaline copper solutions. This method was introduced by Talbot *et al.*, (1945) and has been studied recently by Cope and Bain (1951), and by Robinson and Norton (1950–51).

All these methods include considerable quantities of inactive substances in addition to the active corticosteroids. The figures for the normal ranges are different. Talbot *et al.*, suggested partitioning between benzene and water and considered the portion of neutral reducing steroids entering the water more significant in Addison's disease. At present, however, it appears doubtful whether such fractionation gives any more information than measurement of the total amount of reducing substances as determined by these methods. Methods for corticosteroids have been reviewed by Venning and others (see Venning (1950) ).

## Determination of Urinary Corticosteroids

**Copper Reduction Method for Total Neutral Reducing Steroids** (Cope and Bain (1951) )

The steroids are extracted after acidifying to *p*H 1 and standing for fifteen to eighteen hours by the method suggested by Talbot *et al.*, (1945). Reduction of Nelson's copper reagent is used, the amount of reduction being measured colorimetrically after adding Nelson's arsenomolybdate reagent.

**Reagents.** 1. Chloroform, redistilled.

2. Methyl alcohol.

3. Concentrated sulphuric acid.

4. N/10 sodium hydroxide.

5. Copper reagent (Nelson, (1944) ). Prepare on the day of use by mixing 25 parts of solution A and 1 part of solution B.

Solution A. Dissolve 25 grams of anhydrous sodium carbonate, 25 grams of Rochelle salt, 20 grams of sodium bicarbonate, and 200 grams of anhydrous sodium sulphate in about 800 ml. of water and dilute to 1 litre. Filter if not clear. Keep at not less than 20° C.

Solution B. Dissolve 15 grams of copper sulphate ($CuSO_4$, $5H_2O$) in water and make up to 100 ml.

6. Nelson's arsenomolybdate reagent (see p. 34).

7. Standard solution of desoxycorticosterone acetate, 20 mg. per 100 ml. of methyl alcohol.

**Technique.** Collect a twenty-four-hour specimen of urine without using a preservative. Measure out from $\frac{1}{4}$ to $\frac{1}{8}$ of this and adjust to *p*H 1 with concentrated sulphuric acid. Thymol blue can be used, taking to the colour of the extreme acid end of the range. Allow the acidified urine to stand at room temperature overnight (fifteen to eighteen hours). Extract four times in a separating funnel with redistilled chloroform, each time using a volume equal to about 15 per cent. of that of the volume of urine taken. Note the total volume of chloroform used. Centrifuge the combined chloroform extract which is partly emulsified. Remove the clear chloroform layer and measure its volume, so that a correction can be applied for chloroform extract not separated. Evaporate this clear chloroform extract to about 30 ml. in an all-glass apparatus under reduced pressure, keeping the water in the water bath below 50° C. Transfer the residue quantitatively, washing out with small amounts of chloroform, into a 100 ml. separating funnel. Wash three to four times with 5 ml. portions of N/10 sodium hydroxide and then three times with 5 ml. of water. Back extract each washing with 2 to 3 ml. of chloroform and add this to the main chloroform extract. Next evaporate the washed chloroform extract to dryness under reduced pressure as above. Remove traces of chloroform by adding successively three 5 ml. portions of methyl alcohol and evaporating these under reduced pressure.

Dissolve the dry brownish residue in 2 ml. or more of methyl alcohol. The most satisfactory volume to use is that which gives 100 to 150 $\mu$g. of reducing substance in 0·5 ml. of solution. In the case of normal urines 0·5 ml. of extract should correspond to 1/20th to 1/40th of the daily urine volume so that about 2 ml. of methyl alcohol is required.

Set up the following, using Folin sugar tubes (p. 31).

1. Test, in triplicate. 0·5 ml. of extract, 1 ml. of water, and 1 ml. of copper reagent.

2. Blank. 0·5 ml. of methyl alcohol, 1 ml. of water, and 1 ml. of copper reagent.

3. Standard, in duplicate. 0·5 ml. of standard solution containing 100 $\mu$g. of desoxycorticosterone acetate. 1 ml. of water, and 1 ml. of copper reagent.

Stopper lightly and heat for twenty minutes in boiling water. Cool in running water and then add 1 ml. of the arsenomolybdate reagent. Stand for ten minutes, shaking occasionally, and then add

5 ml. of water to each tube. Mix well and filter through a sintered glass filter. This removes the variable amount of turbidity which is often present and interferes with the colorimeter reading. A filter paper should not be used, since this adsorbs some of the blue colour. Read in the photoelectric colorimeter using a red filter or transmission at 680 millimierons.

**Calculation.** Mg. total neutral reducing steroids per twenty-four hour urine

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}1 \times \frac{\text{Volume of twenty-four hour urine}}{\text{Volume of urine extracted}} \times \frac{\text{Total volume of chloroform used in extraction}}{\text{Volume of clear chloroform layer}} \times \frac{\text{Volume of methyl alcohol solution}}{0{\cdot}5}.$$

INTERPRETATION

The daily excretion of corticosteroids by this method ranges from 2 to 6 mg. This is considerably higher than the figures obtained by other methods. Thus using the phosphomolybdic acid reduction method, Heard, Sobel and Venning found a normal range from 1·0 to 2·1 mg. This difference appears to arise from variations in the method of hydrolysis. Cope and Bain unlike earlier workers allow the acidified urine to stand fifteen to eighteen hours. Robinson and Norton, who allow to stand for twenty-four hours, also get higher values than the earlier workers. Heard and Sobel acidified to *p*H 1, but extracted immediately, whilst Talbot *et al.*, extracted immediately without acidifying. Acid hydrolysis on standing has been shown to increase the amount of non-biologically active metabolites.

Increases have been observed in Cushing's disease and in acromegaly. Injection of adrenocorticotrophic hormone of the pituitary produced a marked increase, 50 mg. by 100 per cent., 100 mg. by over 300 per cent. Very low figures were not obtained in Addison's disease, though lower average values were found. Cope and Bain suggest that total neutral reducing steroids represent metabolites, while excretion of biologically active hormone gives a measure of unmetabolized hormones present in the blood.

Determination of corticosteroids is at present still in the experimental stage and gives little additional information to that which can be obtained from other sources. Much work, however, is in progress. A different method of hydrolysis has been developed recently using the enzyme $\beta$-glucuronidase (Talalay, Fishman, and Huggins, 1946 ; Fishman and Talalay, 1947 ; Marrian, 1950–51 ; Bayliss, 1952 ; Cope and Hurlock, 1952). It appears from work done so far that this gives much higher yields than any of the methods referred to above.

Porter and Silber (1950) used the colour reaction of phenylhydrazine hydrochloride in sulphuric acid, with cortisone and similar compounds with a hydroxyl group on carbon atom 17 (see also Cope and Hurlock). Burton, Zaffaroni, and Keutmann (1951) studied the chromatographic separation of corticosteroids.

See also addendum 2, p. 458.

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*Addenda.* 1. P. 438. Hamburger (1952, *Acta endocrinol.*, 9, 129) brings 50 ml. of absolute alcohol to the boil, adds 40 mg. of ascorbic acid and 10 grams of finely powdered potassium hydroxide, stirs to dissolve for no more than a minute, cools, filters through a sintered glass filter, standardizes and adjusts, and stores in a refrigerator in small bottles.

2. P. 456. Some of the corticosteroids can be oxidized to 17-ketosteroids. Norymberski *et al.* (1953, *Lancet*, 1, 1276) determines 17-ketosteroids in urine before and after oxidation with sodium bismuthate. The difference, termed 17-ketogenic steroids, gives a measure of the corticosteroid excretion.

*CHAPTER XXIV*

# CHEMICAL EXAMINATION OF CEREBROSPINAL FLUID

In addition to the cell count, bacteriological examination, and Wassermann reaction, the following chemical tests are commonly carried out on cerebrospinal fluid : determination of glucose, chlorides, and protein, qualitative test for globulin, and the Lange colloidal gold curve. It is only rarely that other tests such as estimation of urea and calcium are requested. The concentration in the cerebrospinal fluid of certain drugs such as the sulphonamides may sometimes be required.

The fluid ordinarily examined is the lumbar fluid, but occasionally cisternal and ventricular fluids are taken. Any differences between these fluids will be indicated under the various constituents.

Before considering the methods used in examining these fluids it will be useful to say something about their appearance.

## Appearance

Normal cerebrospinal fluids are clear and colourless and give no coagulum or sediment on standing, if they are kept sterile. Abnormalities in appearance may arise in regard to colour, turbidity, and the presence of a coagulum.

**Colour.** The presence of blood is the main cause of an abnormal colour. Normally no red blood cells should be present. Some may be introduced as a result of trauma whilst obtaining the fluid. In such cases, the first few drops should be the most contaminated so that if the first ml. or two is collected separately, the subsequent fluid should be almost if not completely clear. Centrifuging will reveal the presence of small numbers of R.B.C.'s not easily visible to naked eye inspection. They are, of course, seen on microscopic examination.

Pathologically, blood may be present in subarachnoid hæmorrhage and in hæmorrhage into the ventricles. Such blood is more homogeneously mixed with the fluid than is that introduced as a result of trauma, so that there is not the same disappearance of red cells after the first ml. or two of fluid has been withdrawn. Furthermore, hæmolysis often occurs and the hæmoglobin thus liberated is ultimately converted into bilirubin, so that within a few days after such a hæmorrhage, the supernatant fluid obtained on centrifuging, becomes coloured yellow to a depth out of proportion to the number of red cells present. These are in fact disappearing and may have disappeared completely. The colour of fluids from the lumbar region in cases in which there is a complete block due to spinal tumour is also often yellow. The term *xanthochromia* is given to such yellow fluids.

**Turbidity.** Turbidity is seen when there is marked increase in the number of cells or when organisms are present and so is found in meningitis, more especially in the coccal forms. Fluids from cases of tuberculous meningitis are not as a rule turbid. At least 400 to 500 polymorphs per c. mm. are needed to give visible turbidity. Highly purulent fluids may be obtained, for example, in streptococcal meningitis but have been less common since the introduction of the sulphonamides and penicillin. Small numbers of red cells may also give fluids an opalescent appearance. If traces of substances such as alcohol are mixed with the fluid during its collection some opalescence may result.

**Coagula.** Fibrin clots may form, on standing, in pathological fluids containing fibrinogen, which is usually only found when there is a considerable increase in protein. The fluids with a very high protein content obtained in some cases of spinal tumour may set solid on standing. In tuberculous meningitis a fine web-like coagulum sometimes forms if the fluid is allowed to stand overnight. Such a web may take up tubercle bacilli, which are more easily seen if this is examined microscopically. Hence it may be useful, if this is possible, to leave part of the specimen to stand overnight whilst examining the rest immediately.

## GLUCOSE

### Determination of Glucose

This may be carried out by any of the blood sugar methods in use. Since the amount of sugar as thus determined tends to be a little lower in cerebrospinal fluids than in blood, some workers use a larger volume of fluid than is used for blood. This makes for greater accuracy, particularly as the most important abnormality is a reduced glucose content. The methods can easily be adapted to use twice the volume taken in the case of blood. This is perhaps more essential in the case of the titrimetric than with colorimetric methods which are for the most part more suited to the measurement of small amounts of glucose.

#### INTERPRETATION

The sugar content of normal lumbar fluid is usually between 50 and 80 mg. per 100 ml., though a range of 45 to 100 is often allowed. It is thus slightly lower than the blood sugar. The ventricular fluid sugar is rather higher than the lumbar and approximates to the blood sugar.

The most important pathological change is the *decrease* which occurs in meningitis. In meningitis due to the various cocci—meningococci, pneumococci, streptococci, staphylococci—the glucose often disappears completely from the fluid. In tuberculous meningitis, however, although the sugar content is reduced, it is rarely completely absent, being usually between 10 and 40 mg. per 100 ml. In benign lymphocytic choriomeningitis the sugar is often normal, or very nearly so, but may occasionally be decreased to as low as 20 mg.

per cent. In syphilitic meningitis it is almost always within normal limits.

Very small *increases* have been found in some cases of encephalitis and cerebral abscess, but this is of little diagnostic value. Considerable increases are found in diabetes mellitus when the blood sugar is increased, though the blood-sugar is often rather higher than the cerebrospinal fluid sugar. Low fluid sugars are found in hypoglycæmia.

Otherwise, and hence in most conditions in which the cerebrospinal fluid is examined, the glucose content is within normal limits.

Streptomycin can reduce alkaline copper solutions but only does so sufficiently to impair the reliability of glucose estimations if the fluid is taken shortly after intrathecal injection of streptomycin (Smith, 1952).

## CHLORIDES

### Determination of Chlorides

It is possible to titrate most cerebrospinal fluids directly with an appropriate silver nitrate solution using potassium chromate as indicator.

**Reagents.** 1. Silver nitrate solution. Dissolve 2·907 grams of silver nitrate in water and make up to 500 ml. Check at intervals against a standard sodium chloride solution. A 1 per cent. solution is suitable. One ml. of this should require 5 ml. of the silver nitrate solution, since 1 ml. of the silver solution is equivalent to 2 mg. of NaCl and hence to 1 ml. of a 200 mg. per cent. solution of sodium chloride.

2. Potassium chromate, 10 per cent. solution.

**Technique.** Pipette 1 ml. of the cerebrospinal fluid into a 6 × 1 inch test tube containing 2 to 3 ml. of distilled water. Add 2 to 3 drops of potassium chromate and titrate with the silver nitrate to the usual faint brick red colour. Normal fluids require from 3·5 to 3·8 ml.

**Calculation.** Mg. chloride per 100 ml. of cerebrospinal fluid (as NaCl)

$$= \text{ml. of silver nitrate solution required} \times 200.$$

**Notes.** 1. The titration is more accurate with 2 ml. of fluid. However, such an amount of fluid is not always available and it is possible to get quite satisfactory results with 1 ml. If 2 ml. of fluid is used multiply by 100 in the calculation.

2. With really high protein-containing fluids it is advisable to use the blood methods, but this is not often necessary.

3. The methods for blood using 0·2 ml., given on p. 331–2, can be applied and are particularly useful if only a very small quantity of fluid is available.

#### INTERPRETATION

The chloride content of normal cerebrospinal fluid whether lumbar, cisternal or ventricular, lies between 700 and 760 mg. per 100 ml., usually between 720 and 750, and thus is appreciably higher than the plasma chloride. These figures are expressed as sodium chloride.

The most important alteration is in meningitis, when there is a *decrease* usually to between 700 and 600 mg. per cent., occasionally to between 600 and 500. The reduction is generally more marked in tuberculous than in coccal meningitis. It is very unusual for the chloride content to be below 600 except in tubercular meningitis and even in this condition such low figures are only found, as a rule, late in the disease. Very occasionally, even in tuberculous meningitis, the chlorides may be almost normal. In benign lymphocytic choriomeningitis chlorides are usually normal ; there is never more than a slight fall. In all other diseases of the nervous system chlorides are within normal limits. An *increase* is sometimes found in hypertension and in renal disease.

A marked reduction in plasma chlorides in diseases not involving the central nervous system is also accompanied by a low concentration of chloride in the cerebrospinal fluid.

## PROTEINS

### Determination of Total Proteins

Turbidimetric methods are most commonly used though some of the colorimetric methods for estimating proteins have been applied.

#### I. Method of Kingsbury, Clark, Williams, and Post

The turbidimetric method of Kingsbury et al. (1926) is widely used. (See also King and Haslewood, 1936.) The fluid is treated with salicylsulphonic acid and the resulting turbidity compared with standard tubes.

**Reagent.** Salicylsulphonic acid, 3 per cent. solution.

**Preparation of Standard Tubes.** These tubes contain permanent suspensions of formazine in gelatin. Dissolve 65 grams of pure gelatin in 500 ml. of water at about 90° C. Stir into this solution the white of one egg mixed with about twice its volume of water. Heat the mixture on a water bath for an hour, stirring continuously. Filter through a large coarse filter paper using a hot funnel. Keep the clear filtrate at 50° C.

To prepare the formazine add 25 ml. of a 10 per cent. aqueous solution of hexamine to 25 ml. of 1 per cent. hydrazine sulphate. Stopper, shake and leave overnight. Shake the precipitate of formazine which forms so that it is evenly dispersed throughout the liquid. Add 14·5 ml. of this suspension to 100 ml. of the gelatin containing 0·3 ml. of 40 per cent. formaldehyde, which ensures permanent setting. This suspension has been found equivalent to a concentration of 100 mg. protein per 100 ml. of fluid when precipitated as described below. Dilute this suspension with clarified gelatin containing 0·3 ml. of 40 per cent. formaldehyde per 100 ml., in the proportions stated below to obtain a series of standards equivalent to a range of protein from 10 to 100 mg. per 100 ml.

| Tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Protein concentration mg. per 100 ml. fluid . . . | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
| Ml. gelatin-formazine . . | 0·4 | 0·8 | 1·2 | 1·6 | 2·0 | 2·4 | 2·8 | 3·2 | 3·6 | 4·0 |
| Ml. gelatin. . . . | 3·6 | 3·2 | 2·8 | 2·4 | 2·0 | 1·6 | 1·2 | 0·8 | 0·4 | 0 |

Stopper the tubes when cold, cut the corks level with the top of the tubes and dip the stoppered ends in wax. The standards can be checked against serum solutions standardized by a Kjeldahl estimation of the protein nitrogen present. It is recommended that the tubes be mounted in a wooden rack painted black.

Sets of formazine standard tubes can be purchased from Messrs. Gallenkamp, London.

**Technique.** To 1 ml. of cerebrospinal fluid in a small test tube similar to those used for the standards, add 3 ml. of the 3 per cent. salicylsulphonic acid and after standing five minutes compare the turbidity with that of the standards. If the turbidity is greater than that of the 100 mg. per cent. standard, dilute the fluid with saline and repeat.

**Method adapted to the Photoelectric Colorimeter.** Instead of comparing with the above standards the amount of turbidity can be read on the photoelectric colorimeter. To prepare a standard curve, dilute serum with normal saline, determine the protein content by the Kjeldahl method (see p. 165), and adjust to 100 mg. per 100 ml. of solution. Set up from this a series of tubes as follows and add 6 ml. of salicylsulphonic acid to each. Stand

| Mg. protein per 100 ml. C.S.F. | 0 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ml. of solution, 100 mg. protein per 100 ml. . . | 0 | 0·2 | 0·4 | 0·6 | 0·8 | 1·0 | 1·2 | 1·4 | 1·6 | 1·8 | 2·0 |
| Ml. of water . . . | 2·0 | 1·8 | 1·6 | 1·4 | 1·2 | 1·0 | 0·8 | 0·6 | 0·4 | 0·2 | 0 |

for five minutes and read in the colorimeter using a red filter or transmission at 660 millimicrons. The amount of turbidity obtained on mixing 1 ml. of cerebrospinal fluid with 3 ml. of salicylsulphonic acid as described above is read in the same way and the protein content obtained from the standard curve. When the protein content is more than 100 mg. per 100 ml. dilute with saline so as to bring it below this level.

### II. Method of Meztrezet

The protein is precipitated with trichloracetic acid and heat. The turbidity is compared with a series of standard tubes prepared from solutions of known protein content treated in the same way.

**Reagent.** Trichloracetic acid, 30 per cent. (w/v) solution.

**Preparation of Standard Tubes.** Dilute a few ml. of non-haemolyzed serum with normal saline about 1 in 10 and determine the protein

content by one of the methods used for estimating the serum proteins. Then dilute the fluid so that it contains exactly 100 mg. protein per 100 ml. Using this, prepare a series of tubes as follows, scratching the protein content on the tube with a diamond. Take care to obtain a set of tubes of similar cross-section, 5 × ½ inch being suitable.

| Mg. protein per 100 ml. C.S.F. | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 50 | 60 | 80 | 100 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ml. solution containing 100 mg. protein per 100 ml. . | 0·2 | 0·3 | 0·4 | 0·5 | 0·6 | 0·7 | 0·8 | 1·0 | 1·2 | 1·6 | 2·0 |
| Ml. water . . . | 1·8 | 1·7 | 1·6 | 1·5 | 1·4 | 1·3 | 1·2 | 1·0 | 0·8 | 0·4 | 0 |

Add 0·3 ml. of the 30 per cent. trichloracetic acid and heat the tubes carefully in a small bunsen flame, or better, place in a beaker of boiling water for two minutes. Allow to cool and then seal off the tubes. Sterilize by heating on three successive days for one hour in a bath at 56° C.

**Technique.** Take 2 ml. of fluid in a test tube of similar bore to that used for the standards, add 0·3 ml. of 30 per cent. trichloracetic acid and heat as described for the standards. Allow to cool spontaneously and then compare with the standards. Comparison is good up to 50 mg. per 100 ml. and fairly good up to 100 mg. per cent., but above this, fluids must be diluted with saline so as to bring the protein content into a suitable range, preferably to between 20 and 50 mg. per 100 ml.

Although it has been claimed that the standards keep for years unchanged, it is recommended that new standards be made every two to three months. If serum protein estimations are being carried out regularly this can easily be done.

It is obvious that at best turbidimetric methods are only approximate because of the difficulty of producing particles of the same size each time. However, they are simple and quick and have proved to have a degree of accuracy which is satisfactory for clinical purposes. The error is usually less than 10 per cent. but occasionally may be up to 20 per cent.

The colorimetric methods for determining proteins have been applied and adaptations of both the biuret (see, for example, Andersch, 1944 ; Sundermann and Schmidt, 1944), and tyrosine methods can be used. In addition to estimating the total proteins, differential estimation of albumin and globulin can be made by these methods but is very rarely done.

### INTERPRETATION

The protein content of normal lumbar fluids lies between 15 and 40 mg. per 100 ml., most containing from 25 to 30 mg. per 100 ml. The protein present is almost entirely albumin. Cisternal fluid has on the average a slightly lower protein, being most often in the region of 20 mg. per 100 ml. In the case of ventricular fluids it is appreciably lower, being usually only 10 mg. per cent.

An increase in the total protein is the commonest abnormality

found. The protein in such cases is a mixture of albumin and globulin with the former greatly preponderating. The globulin tests described below give a rough index of the globulin increase. As we have seen, fibrinogen may be present in some fluids in which there is a considerable increase in protein and may give rise to a clot on standing.

An indication of the extent to which the proteins are increased in various diseases is given later in this chapter. It is seen that in many conditions the increase is small, rarely exceeding 100 mg. per cent., though increases of up to 400 mg. per 100 ml. are not infrequent in the different types of meningitis, in polyneuritis, and in tumours. Values higher than this are rare, though occasionally in spinal tumour in which there is a complete block, lumbar fluid may have a protein content of up to several grams per 100 ml. and may thus approach that of plasma.

It is useful to note those conditions in which, on the one hand, the protein increase is associated with an increase in cells, and those, on the other hand, in which the cell count is normal. Cell increase with protein increase is found essentially in inflammatory lesions and is seen in all forms of meningitis, in tabes dorsalis and general paralysis of the insane, and in poliomyelitis. Small increases in cells may occur in polyneuritis and in disseminated sclerosis, not always with a protein increase in the latter case. Isolated increase in proteins is found most commonly in tumours but may also be found in neuritis and in cerebral arteriosclerosis.

### Qualitative Tests for Globulin

Two tests are in common use for showing the presence of an increase in globulin in cerebrospinal fluid, the Nonne-Apelt and the Pandy tests.

#### 1. Nonne-Apelt Test

The globulin is precipitated by half-saturating with ammonium sulphate.

**Reagent.** Saturated ammonium sulphate solution. Dissolve 85 grams of ammonium sulphate in 100 ml. of hot distilled water, allow to cool overnight and filter. Keep in a well-stoppered bottle.

**Technique.** Add 1 ml. of this saturated solution to 1 ml. of fluid whilst shaking. Smaller volumes may be used if desired so long as equal volumes of the two are mixed. Stand for three minutes and read the amount of opalescence or turbidity by inspection. Report findings as, no opalescence, slight opalescence, opalescence, marked opalescence, and turbidity. Almost all normal fluids remain quite clear and never show more than the faintest suspicion of opalescence.

The test has been carried out by layering the cerebrospinal fluid on to the saturated ammonium sulphate and noting whether a white ring appears where the liquids meet. This method is open to the objection that there may be appreciably more than half saturation with the sulphate at the junction of the fluids.

### 2. Pandy's Test

In this test a saturated phenol solution is used.

**Reagent.** Pandy's solution. Prepare by dissolving 10 grams of phenol in 150 ml. of distilled water. The solution should be clear and colourless.

**Technique.** Add 2 drops of cerebrospinal fluid to 2 ml. of Pandy's solution. Most normal fluids show no opalescence at all and none more than the faintest degree of opalescence. The test is reported as showing no opalescence, slight opalescence, opalescence, marked opalescence and turbidity. The test has the advantage of requiring only 2 drops of fluid and is in fact rather more sensitive than the Nonne-Apelt.

INTERPRETATION

These tests give a rough idea of the extent of any increase in globulin. Taken in conjunction with the estimation of the total protein they enable an approximate comparison to be made between the globulin increase and the increase in total proteins. There is a more marked increase in globulin for a given rise in total proteins in conditions such as general paralysis of the insane, tabes dorsalis and disseminated sclerosis than there is in most other conditions involving the central nervous system. In tabes and in disseminated sclerosis a positive globulin test can be obtained with fluids which show no increase in total proteins.

### Lange Colloidal Gold Curve

This test depends on the precipitating action of some of the globulin fraction in abnormal cerebrospinal fluids on a colloidal gold solution under controlled conditions.

**Preparation of the Gold Sol.** Method of Maclagan (1946)

**Reagents.** 1. One per cent. gold chloride solution. Dissolve 1 gram of sodium gold chloride, $NaAuCl_4$, in double distilled water, and make up to 100 ml. with the same water.

2. Sodium citrate, 1·6 per cent. solution. Make up in double distilled water.

3. Double distilled water, for making up the solutions and the gold sol. Redistil ordinary distilled water from an all-glass still into a glass vessel. Pyrex glassware is suitable and may be used throughout the test.

**Technique.** The gold sol can be made in any desired amount. Bring the double distilled water to a brisk boil in a suitable flat-bottomed flask and add 1 ml. of 1 per cent. gold chloride and 1 ml. of 1·6 per cent. sodium citrate solution per 100 ml. of water. After boiling for a few seconds reduce the flame and simmer gently until the series of colour changes which occurs is complete. These proceed from a bluish colour to a dark purple to a final clear red. Place a filter funnel in the mouth of the flask during this simmering to reduce the loss by evaporation. When the red colour is reached cover the

mouth of the flask and allow to cool. The solution keeps well for some weeks if well protected and if pipettes are not introduced directly into it. It is, however, safer to prepare freshly every few days and this should be done if convenient.

### Oxalate Method of preparing the Gold Sol

**Reagents.** 1. One per cent. gold chloride solution as above.
2. One per cent. neutral potassium oxalate solution.
3. One per cent. potassium hydroxide solution.
4. Double distilled water as above for all solutions.

**Technique.** Measure out 100 ml. of double distilled water into a 250 ml. Pyrex flask and add 1 ml. of the oxalate solution and 0·32 ml. of the hydroxide. Bring to the boil and whilst boiling add 1 ml. of the gold solution, drop by drop. Continue boiling for a few seconds and then allow to cool. The solution as above should be clear cherry red without any fluorescence. The amount of hydroxide needed has to be determined empirically but in the author's experience is fairly uniformly found to be as stated for good quality sodium gold chloride.

All glassware should be thoroughly cleaned before use. Wash with acid, steam, wash with distilled water and allow to stand filled with double distilled water until used.

There is still a certain fortuitous element in the preparation of satisfactory gold sols. See, for example, Maclagan's experience with corks (Maclagan, 1946). The author had a similar experience. Furthermore, differences may be observed between distilled water from different stills. Thus, better results may sometimes be obtained with once distilled water than with double distilled.

### Technique for the Lange Gold Curve

A series of dilutions of cerebrospinal fluid are prepared and gold sol added to each. Any colour change is noted.

(*a*) **Using Sodium Chloride as Diluent. Reagents.** 1. Gold sol. Prepare as above. In the case of the citrate method add 1·33 ml. of 0·02 N sodium hydroxide to each 100 ml. of solution. This quantity, which is recommended by Maclagan has also been found satisfactory by the author, but may be altered in the light of experience. The oxalate sol prepared as described, if satisfactory in colour is ready for use.

2. Sodium chloride, 0·26 per cent. solution. This can be prepared by diluting a 4 per cent. solution just before use.

**Technique.** Prepare a rack of ten clean test tubes—five by $\frac{5}{8}$ inch tubes are suitable. Clean by washing as described above.

Pipette 1·8 ml. of the salt solution into the first tube and 1 ml. into each of the remaining tubes. Add 0·2 ml. of cerebrospinal fluid to the first tube, mix well, withdraw 1 ml. of the mixture and add to tube two. Mix and again withdraw 1 ml. and add this to tube three. Proceed in this way along the tubes, finally discarding 1 ml. from the last tube. Then add 5 ml. of the gold sol to each tube, mix, and allow to stand overnight. The results are read using a series of numbers to represent the colour changes, as follows :

0. No appreciable change from the original cherry red.
1. A slight change to purplish red.
2. Purple.
3. A more bluish purple.
4. Pale blue or bluish purple with some precipitation.
5. Complete precipitation giving a deposit of the gold at the bottom of the tube with a clear colourless liquid above.

There is a certain amount of qualitative variation in the shades of colours obtained from case to case but when familiar with the colours little difficulty is experienced in reading the results.

The test can be done using half the above quantities if desired, that is, 0·9 ml. of salt in the first tube, 0·5 ml. in the other tubes, 0·1 ml. of fluid and 2·5 ml. of gold sol.

In many cases only six tubes need be used, thus economizing gold sol. As a routine seven tubes can be set up so that after transferring 1 ml. from tube six to seven, the 2 ml. of fluid can be left in tube seven and gold sol added to the first six tubes. Then, if there is any colour change in tube six, the test can be extended to the whole ten tubes. If no colour change has occurred by the end of an hour, the test is almost certainly negative. For full precipitation to stage five to occur, standing overnight is necessary.

(*b*) **Using Phosphate Buffer** (Maclagan). **Reagents.** 1. Gold sol prepared as described under the citrate method. No addition of sodium hydroxide is made.

2. Phosphate buffer, *p*H 6·6. Dissolve 0·314 gram of anhydrous potassium dihydrogen phosphate and 0·207 gram of anhydrous disodium hydrogen phosphate in water and make up to 100 ml. Prepare fresh if any moulds begin to grow in it.

**Technique.** Tubes are put up exactly as described above except that the phosphate buffer is used instead of the salt solution. Read in the same way.

INTERPRETATION

Normal fluids produce little or no change. No more than a slightly bluish-red colour in tubes three to five should result, giving at the most, say, 0011000000.

Three types of abnormal curve have been recognized.

1. The *paretic* curve, characterized by complete precipitation in the first few tubes, for example : 5554321000.

2. The *tabetic* or *luetic* curve, in which there is maximum change in colour in tubes three to five, but complete precipitation does not occur in any tube. An example is 1233210000.

3. The *meningitic* curve, in which the maximum colour change occurs in tubes five to eight, for example, 0001233210.

As the name implies, the paretic curve is found most typically in general paralysis of the insane and the luetic curve in tabes dorsalis. One or other of these types of curve or one intermediate between them is obtained in cases of syphilis of the central nervous system. In addition similar curves of paretic or near paretic type are obtained in some cases of disseminated sclerosis. The meningitic curve is

sometimes, but by no means always, obtained in meningitis. Mildly tabetic curves showing changes up to about 2 are sometimes obtained in encephalitis, whilst mildly meningitic curves are occasionally seen in conditions where there is a very high protein. The writer has seen, though very rarely indeed, paretic curves in a few patients with other diseases for example, in cerebral tumours, and in tuberculous meningitis treated for a long period with streptomycin, but with very few exceptions such curves are obtained only in tertiary syphilis and in disseminated sclerosis.

It will be seen that the test is only of limited value. With a normal cell count, normal proteins and no globulin increase, it can be said that negative findings are 100 per cent. Positive results are also not often obtained with fluids in which the cell count is normal and protein is increased, and when they are, the change is almost always only slight to moderate. In tertiary syphilis, the cell count and protein changes are fairly characteristic and the Wassermann reaction is almost always positive, but the test does yield further confirmatory evidence and may be useful in following the effects of treatment. Fluids are brought almost completely back to normal by the penicillin treatment of these conditions. The test may, however, be of considerable diagnostic value in disseminated selerosis. It is of negligible value in meningitis.

The most generally accepted explanation of the abnormal curves is that they result from an increase in the globulin fraction of the protein, particularly in the $\gamma$-globulin, or using the Howe fractionation the euglobulin. This globulin fraction, by neutralizing the electric charge on the particles of the gold sol, causes an increase in their size and so produces the series of colour changes noted and ends in some cases with particles large enough to settle out within twenty-four hours. Albumin on the other hand, has a protective action. Accordingly, positive curves are obtained when the increase in the type of globulin responsible is proportionately greater than is the increase in albumin. The greatest such relative increase occurs in general paralysis of the insane and in disseminated selerosis, then in tabes dorsalis, and still less in those cases of meningitis which give a meningitic curve. Normal curves are obtained when the increase in albumin is great enough to counter any increase in the globulin. Lange, however, has suggested that abnormal proteins may be present in general paralysis and be responsible for the paretic curve.

**Note.** It is helpful in carrying out the test to keep a cerebrospinal fluid known to give a paretic curve so that this can be used to test batches of the gold sol when desired. Such fluids frozen in the refrigerator keep well for months. Adequate normal controls should also be used.

It is only rarely that any other estimations are carried out. The **urea** content follows the blood urea fairly closely though it may be a little lower when the blood urea is high, particularly when it is rising. Estimation of the urea content gives no information which the blood urea does not give but may be done in cases of coma in which the

cerebrospinal fluid has been withdrawn, and so make it unnecessary to take a blood specimen as well. Methods of estimation are identical with those used for blood.

The **calcium** content of the fluid is much lower than that of blood serum because of the much lower protein content. The normal range is 5·5 to 6 mg. per 100 ml. of fluid. It is reduced in tetany due to a decrease in the physiologically active calcium of the plasma. The calcium can be estimated in the same way as the serum calcium but it is better, if possible, to take a larger volume of fluid, say 3 or 4 ml.

Special tests have sometimes been used on cerebrospinal fluids. They are positive in tuberculous meningitis and mostly negative in other conditions but are not entirely specific.

**The Tryptophane Test,** so-called because the colour obtained is said to be due to the presence of tryptophane groups, free or combined.

**Reagents.** 1. Concentrated hydrochloric acid.

2. Formaldehyde, 2 per cent. Dilute 40 per cent. formalin 1 in 20.

3. Sodium nitrite, 0·06 per cent. solution.

**Technique.** To 2 to 3 ml. of the cerebrospinal fluid in a suitable test tube add 15 ml. concentrated hydrochloric acid and 2 to 3 drops of 2 per cent. formaldehyde. Shake and allow to stand for four to five minutes. Carefully run in about 2 to 3 ml. of 0·06 per cent. sodium nitrite so as to get a sharp line of demarcation. When the reaction is positive, a delicate violet ring is formed at the junction of the fluids. This may take a few minutes to become visible. If the reaction is negative there is either no colour or a yellow ring. If in doubt consider the test negative. A positive reaction may be obtained in tuberculous meningitis, with blood-stained purulent fluids, and with xanthochromic fluids whatever the condition.

The test is of limited value but may occasionally give a little support one way or the other in more doubtful cases of tuberculous meningitis.

**Levinson's Test. Reagents.** 1. Mercuric chloride, 2 per cent. aqueous solution.

2. Salicylsulphonic acid, 3 per cent. solution.

**Technique.** Take two small test tubes 3 × $\frac{3}{8}$ and pipette 1 ml. of cerebrospinal fluid into each. To one add 1 ml. of the mercuric chloride solution and to the other 1 ml. of the salicylsulphonic acid. Stopper, mix, and stand in a rack for twenty-four hours. Measure the height of the two deposits. With normal fluids these are both less than 2 mm. A heavy sediment forms if there is an increase in total protein. The mercuric chloride one is light and feathery, forming slowly as compared with the salicylsulphonic acid one, which, forming quickly, is dense and heavy.

In tuberculous meningitis the mercuric chloride sediment is more than 2 mm. and is two or more times greater in height than the other, whereas in coccal meningitis the deposit in the salicylsulphonic acid tube is the greater.

The findings in normal fluids may be summarized as follows :

TABLE XXX

*Composition of Normal Cerebrospinal Fluid*

| | |
|---|---|
| Cells | 0 to 4 mononuclears per c.mm. |
| Pressure | 80 to 150 mm. of water. |
| Colour and appearance | Clear and colourless. No coagulum or deposit. |
| *p*H | 7·4 to 7·6. |
| Specific gravity | 1·006 to 1·007. |
| Protein content | 15 to 40 mg. per 100 ml. (Lumbar). |
| Globulin | Nonne-Apelt and Pandy tests negative. |
| Glucose | 45 to 100 mg. per 100 ml. |
| Chlorides | 700 to 760 mg. per 100 ml. (as NaCl). |
| Urea | 20 to 40 mg. per 100 ml. |
| Calcium | 5·5 to 6 mg. per 100 ml. |
| Cholesterol | Trace only. |
| Inorganic phosphorus | 1·5 to 2·1 mg. per 100 ml. (as P). |
| Lange curve | None given. |

## Summarized Findings in Disease

It is thought that a summary of the findings in the more important diseases of the central nervous system may usefully be given here. The variations in cell count and pressure are included so as to give a more comprehensive picture. For a full discussion of this subject see Greenfield and Carmichael (1925).

**Meningitis.** The pressure is increased in all forms of meningitis to 300 to 1,000 mm. of cerebrospinal fluid.

**Coccal Meningitis.** The fluid is usually opalescent or purulent in appearance. There is marked cell increase, almost entirely polymorphonuclear, generally to over 1,000 per c.mm. Total protein is much increased, most often to between 100 and 400 mg. per 100 ml., globulin tests being positive. Chlorides are decreased to between 600 and 700 mg. per 100 ml., whilst glucose is often completely absent. The causal organism can usually be isolated, the meningococcus being the most difficult to grow.

**Tuberculous Meningitis.** The fluid is usually clear and colourless though it may become tinged yellow in the later stages. A fine fibrin clot may form if the fluid is allowed to stand undisturbed for twenty-four hours, and may contain tubercle bacilli. The cells, which in most cases are between 100 and 300 per c.mm., are a mixture of lymphocytes and polymorphonuclears with a greater proportion of the former in the majority of fluids. Proteins are appreciably increased, generally being between 50 and 400 mg. per cent. Chlorides are decreased, ranging from normal to 500 mg. per 100 ml., under 600 being almost diagnostic. The glucose content falls but it is rare for it to be completely absent, a figure round about 30 mg. per cent. being commonly found, with a range of 10 to 45.

**Serous Meningitis, Benign Lymphocytic Choriomeningitis.** The fluid is clear and colourless with an almost purely lymphocytic cell

increase, which whilst it may be considerable and exceed 1,000 per c.mm., is often moderate between 50 and 500. Proteins also show in most cases only a relatively moderate increase to between 50 and 100 mg. per 100 ml., whilst chlorides and glucose are often normal or but slightly reduced. However, cases have been reported in which there was an appreciable fall in glucose down almost to 20 mg. per cent.

**Neurosyphilis.** Clear colourless fluids without coagula are the rule. The pressure may be slightly increased, more particularly in G.P.I., to 200 mm. of cerebrospinal fluid. Cells are usually found to be between 10 and 100 per c.mm., only very occasionally exceeding 100, whilst the proteins are between 30 and 100 mg. per 100 ml., again only rarely exceeding this figure. Increases in both cells and proteins are more marked on the average in general paralysis than in tabes dorsalis though there is considerable overlapping. Globulin tests are positive, the relative increase in comparison with the increase in total proteins being more marked than in almost all other diseases of the central nervous system. It is also greater in general paralysis than in other types of neurosyphilis. Positive Lange curves are obtained, paretic curves in G.P.I. and tabetic in tabes dorsalis and other cases of neurosyphilis. Chlorides and glucose are almost always within normal limits. According to Greenfield and Carmichael the Wassermann reaction is positive in 100 per cent. of cases of general paralysis and in approximately 70 per cent. of cases of tabes dorsalis.

**Disseminated Sclerosis.** Clear colourless fluids are found with an occasional slight increase in pressure. Many fluids are completely normal. Increase in cells when present is usually slight, from 4 to 10 per c.mm. Only rarely does the cell count exceed 10 though very occasionally it may be over 50. The cells are lymphocytes only. Proteins are also usually normal, values of more than 45 mg. per cent. being found in only a small minority of cases. Weakly positive globulin tests are frequently given even when the total proteins are within normal limits. Paretic and luetic Lange curves are often obtained. As Greenfield and Carmichael (1925) pointed out, the association of a paretic type of Lange curve with a slight increase in cells, little or no increase in proteins, but with weakly positive globulin tests and negative Wassermann reactions in cerebrospinal fluid and blood is almost diagnostic of this condition.

**Encephalitis Lethargica.** The fluid is clear and colourless, but the pressure is raised to over 200 mm. Cells show an increase but do not often exceed 100 per c.mm. and are all lymphocytes. Proteins are often normal but may be slightly increased. Normal or mildly luetic Lange curves may be obtained. Glucose may be high in the normal range or even be slightly above the upper normal limit, that is within the range 80 to 120 mg. per 100 ml. Chlorides are normal.

**Poliomyelitis.** Though the fluid is clear and colourless there is sometimes a coagulum in the later stages. In the earlier phases of the disease there is a cell increase which is often large—30 to 2,000 per c.mm.—and consists mainly of polymorphonuclears. At this stage there is only a small to moderate increase in proteins. Subse-

quently the cell count falls rapidly and becomes chiefly lymphocytic. Total proteins rise as the cell count falls and may reach 200 to 300 mg. per cent. before they begin to return to normal. Glucose and chlorides are within normal limits. Mildly luetic Lange curves may be given.

**Neuritis.** In neuritis involving a single nerve the fluid is generally normal, but there may be a small increase in proteins. Thus, for example, in *sciatica*, the proteins are often found to be between 40 and 70 mg. per 100 ml., and very rarely between 70 and 100.

In *polyneuritis*, toxic or infective, fairly typical findings are met. The fluid is clear and colourless with a small increase in cells which as a rule does not exceed 20 per c.mm. and is lymphocytic. Total proteins are appreciably increased, most often to between 100 and 200 mg. per cent. Globulin tests are positive and a slight to moderate Lange curve may be obtained, for example 0011221100, often reaching 2 but only occasionally 3. Glucose and chlorides are normal.

**Spinal Tumours.** The most frequent finding is a moderate to a very large increase in total proteins, cells being normal or only slightly increased in number. Total proteins may reach and exceed 1,000 mg. per 100 ml., depending on the position and the completeness of the block. When fluid is obtained from below a complete block it may approximate to plasma in its protein content. Pressure is low, the fluid may be yellow in colour and on standing produce a firm fibrin clot. Such an association of a yellow fluid of very high protein content and a coagulum is known as Froin's Syndrome. Fluids taken from above the block have an essentially normal protein.

**Brain Tumour and Brain Abscess.** Very varied findings are obtained in these cases. The position of the lesion and in the case of an abscess the degree of encapsulation is such that in many there is no alteration other than some increase in pressure. Other cases show :

**Brain Tumour.** An increase in proteins without increase in cells is found in some cases ; in others there may be both increase in cells up to about 80 lymphocytes per c.mm. and in total proteins up to 300 to 400 mg. per 100 ml.

**Brain Abscess.** There may be an increase in cells more often moderate in degree but sometimes marked to 1,000 per c.mm. This increase is mainly lymphocytic though containing some polymorphonuclears. A small cell increase in the range 5 to 20 and containing polymorphonuclears is highly suggestive of brain abscess. Proteins are usually found to be normal or to show a moderate increase. Chlorides and glucose show no fall so long as the abscess is encapsulated and does not give rise to meningitis, indeed the glucose may sometimes be found to be high in the normal range or even to be slightly raised. A leaking abscess may, however, lead to a fall in glucose. A progressive fall in chlorides is an indication that meningeal involvement is becoming widespread.

**Cerebral Hæmorrhage and Thrombosis.** These conditions may only lead to a rise in the pressure but are commonly found also to have a

moderate increase in total proteins. In cerebral hæmorrhage blood may become mixed with the fluid which on standing or centrifuging leaves a yellow supernatant fluid. The leucocyte count can be compared with the red cell count in cases of suspected hæmorrhage. As we have seen, differentiation from accidental trauma when taking the specimen is usually satisfactorily made by collecting in two or three tubes. In trauma the first specimen will be more heavily contaminated whereas in hæmorrhage the blood is evenly mixed. It should be noted that leucocytes break down more slowly than red cells and so remain longer in the fluid.

The protein content increases, but only moderately, though often to a greater extent than is accounted for by the contamination with blood. Yellow fluids due to hæmorrhage have a protein content which is usually below 100 mg. per 100 ml., whereas in tumours much higher protein values are found with less yellow fluids.

**Hypertension.** In hypertension it is common to find the total proteins somewhat increased, figures between 50 and 100 mg. per cent. often being seen. It is rare for the proteins to be higher than this.

## MISCELLANEOUS FLUIDS

These include effusions into serous cavities such as the pleura and the peritoneum ; œdema fluids from subcutaneous tissues ; and cyst fluids.

Chemical examination of these fluids is requested much less commonly than in the case of cerebrospinal fluid. It is, however, sometimes useful to know the *protein* content. In health there is almost no protein in the very small amount of fluid present in the serous cavities and tissue spaces, but in disease the protein content can range from below 100 mg. per 100 ml. to a concentration almost equal to that of the plasma. A division is often made into exudates and transudates. This is useful, though there is actually no hard and fast dividing line. The formation of exudates is associated with a considerable degree of inflammation, which by altering capillary permeability allows protein to pass through. As a result exudates have a high protein content, usually above about 3 grams per cent. Fibrinogen is also often present so that the fluid may set solid on standing. On the other hand, the protein content of transudates is lower, since in the absence of inflammation capillary permeability is unaltered, other factors, such as the low plasma proteins in nephrosis, being responsible for causing the œdema. Proteins are often below 1 gram per 100 ml., and in nephrosis, for example, may even be below 100 mg. per 100 ml. In acute nephritis the proteins may be moderately high, often between 1 and 2 grams per cent., according with the view that there is general capillary damage permitting some passage of protein. Absorption of water may lead to increase in the protein content of transudates when the fluid has been present for an appreciable time.

A sufficiently reliable measure of the protein content for most purposes can be obtained by diluting the fluid and applying the

turbidimetric methods given for cerebrospinal fluids. For more accurate results use the methods given for plasma proteins.

The *specific gravity* mainly reflects the amount of protein present. The range is from 1·005 to 1·035. It has been held that the specific gravity is below 1·015 in transudates and above 1·018 in exudates.

The *sugar* content is approximately the same as that of the plasma unless it is reduced by cells and bacteria present. This may be the case with some exudates.

The *chloride* content is related to the protein content. When protein concentration is highest as in severe inflammatory exudates chloride content approximates to that of the plasma, but in transudates of very low protein content chloride concentration is higher and resembles that of cerebrospinal fluid.

There is little if any *fat* in normal fluids. The milky fluids sometimes obtained are of several types. True chylous fluids result when admixture with chyle from the thoracic duct occurs. Several per cent. fat may be present. Fluids which contain fat formed from cell degeneration are described as chyliform. Some milky or opalescent fluids may contain little fat, the appearance being due to cholesterol, lecithin, or even to protein. Such fluids are termed pseudochylous.

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## *CHAPTER XXV*

# MILK

THE hospital biochemist is sometimes asked to analyse specimens of milk, both ordinary cows' milk and human milk. Lactose, fat, and total nitrogen are the constituents most frequently estimated.

### Determination of Lactose

Lactose may be determined either by using Benedict's Quantitative solution as used for estimating urine sugar, or by one of the micro-methods for estimating blood sugar.

#### 1. Using Benedict's Quantitative Solution

**Reagents.** 1. Sodium tungstate, 10 per cent. solution.
2. Sulphuric acid, 2/3 N.
3. Benedict's quantitative solution, see p. 69.

**Technique.** Pipette 10 ml. of milk into a 100 ml. volumetric flask and add 5 ml. of 10 per cent. sodium tungstate and 5 ml. 2/3 N sulphuric acid. Mix well, stand for a few minutes, and make up to the mark with water. Mix well and filter. Smaller quantities of milk may be used if necessary, keeping the proportions of the reagents the same. In the case of cows' milk it is more convenient to use more milk and take proportions of 20 ml. milk with 12 ml. of tungstate and acid because of the different concentrations of lactose and protein. After filtering, run the filtrate, which should be quite clear, into a burette and titrate 25 ml. of Benedict's quantitative solution in exactly the same way as is described for urine sugar, p. 70.

**Calculation.** Twenty-five ml. of Benedict's quantitative solution are reduced by 67 mg. of lactose. Hence

Grams lactose per 100 ml. milk

$$= \frac{\text{Number of times the milk was diluted}}{\text{Ml. required for titration}} \times \frac{67 \times 100}{1{,}000}$$

$$= \frac{67}{\text{Ml. required for the titration}}$$

if 10 ml. of milk was diluted to 100 ml. The dilution is 1 in 10 for human milk, 1 in 5 for cow's milk.

#### 2. Using Micro-methods for Blood Sugar

A dilution of 1 in 25 or 1 in 50 is convenient and this can then be used with any of the blood sugar methods given earlier in Chapter II. The method should be standardized against lactose solutions of known strength, either 100 or 200 mg. per 100 ml.

### Determination of Fat

Concentrated sulphuric acid is added to the milk. Whilst the fat is unaffected, other organic matter present is charred. Centrifuging

then brings the fat into a calibrated part of the tube used so that the amount of fat present can be read directly. Several different types of tube are in use. One of the most frequently used is the Babcock tube (Fig. 69).

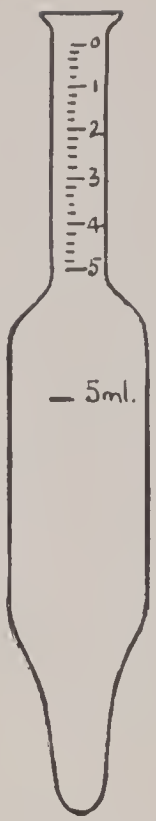

Fig. 69. Babcock Fat Tube.

**Reagents.** 1. Concentrated sulphuric acid (S.G. 1·83 to 1·84).

2. Acid alcohol mixture. Mix equal volumes of concentrated hydrochloric acid and amyl alcohol.

**Technique.** Run in 5 ml. of milk. Add sulphuric acid in approximately 1 ml. amounts at short intervals until the bulb of the tube is filled to the top. Mix well by rotation after each addition. Cool, and then add further acid so as once again to fill the bulb. Then without mixing add sufficient of the acid alcohol mixture to bring the level up to the zero mark in the neck. Centrifuge. Read the amount of fat directly on the calibrated part of the neck. If necessary bring the upper layer of the fat to the zero mark by addition of warm water and recentrifuging. Should the fat content be in the region of 5 per cent. or over, dilute the milk 1 in 2 with distilled water and proceed as above, multiplying the observed reading by 2.

## Determination of Protein

### 1. Modified Esbach's Method (Bogg's)

**Reagent.** Bogg's reagent. Dissolve 50 grams of phosphotungstic acid in 250 ml. of water and add 50 ml. of concentrated hydrochloric acid and mix. Keep in a dark glass bottle.

**Technique.** Dilute the milk 1 in 10 with distilled water in the case of human milk and 1 in 20 with cows' milk. Fill the Esbach tube to the U mark with the diluted milk and then up to the R mark with Bogg's reagent. Invert several times to mix and allow to stand for twenty-four hours. Read off directly in grams protein per 1,000 ml. of milk. To obtain the grams of protein per 100 ml. milk, divide by 10 and multiply by the number of times the milk was diluted.

### 2. Micro-Kjeldahl Nesslerisation Method

**Reagents.** As for estimation of plasma proteins.

**Technique.** The estimation is carried out in the same way as for plasma proteins, but using a different dilution since milk has a smaller protein content. For cows' milk use a dilution of 1 in 25, for human milk one of 1 in 15 is convenient. Otherwise carry out as there described (p. 165), but instead of using 6·25 as the factor for converting nitrogen into protein use 6·38.

The micro-Kjeldahl method and apparatus can be used. Take 0·5 ml. of milk, add 2 ml. of the digestion mixture and after digestion distil over into 25 ml. of N/100 sulphuric acid.

### Total Solids

These can conveniently be determined by evaporating a few grams of milk in a weighed evaporating dish, heating first on a water bath and finally in an air oven at 100° C.

### Phosphatase

Determination of milk phosphatase is carried out to check the reliability of pasteurization. If this has been done efficiently the milk should show no phosphatase activity. The method described for serum alkaline phosphatase can be applied to milk.

**Note.** In examining human milk it is usual either to empty the breast completely, using a breast pump, the most satisfactory time being four to six hours since the previous feed, or to collect a specimen about the middle of a feed. This is done because the composition of the milk varies, becoming more concentrated during the course of the feed.

## Composition of Milk

The major constituents of milk are the carbohydrate, lactose; fats; and proteins. The proteins include caseinogen—a phosphoprotein containing 0·7 per cent. phosphorus—lactalbumin, and a trace of lactoglobulin. In addition milk contains important amounts of calcium, phosphorus, and vitamins A and $B_2$. There are only small quantities of vitamins $B_1$, C and D, and of iron. Milk accordingly is rather deficient in these.

Milk varies widely in composition. Furthermore, there are important differences between human milk and cow's milk. These are summarized in Table XXXI.

TABLE XXXI

*Composition of Milk*

| | Human | | Cows' | |
|---|---|---|---|---|
| | Range (per cent.) | Average | Range (per cent.) | Average |
| Water | 82–90 | 87 | 82–90 | 87 |
| Protein | 1·0–2·0 | 1·3 | 2·5–4·5 | 3·4 |
| Caseinogen | — | 0·8 | — | 2·85 |
| Albumin (including a little globulin) | — | 0·5 | — | 0·55 |
| Fats | 2·0–4·5 | 3·3 | 2·5–5·5 | 3·7 |
| Lactose | 5·6–7·8 | 6·8 | 3·5–5·6 | 4·8 |
| Ash | 0·1–0·4 | 0·25 | 0·6–0·9 | 0·73 |
| Calcium | 0·018–0·042 | 0·030 | 0·09–0·17 | 0·125 |
| Phosphorus | 0·010–0·020 | 0·016 | 0·07–0·12 | 0·095 |

It will be seen that whilst the fat content is approximately the same, there is more lactose but less protein, calcium, and phosphorus in human than in cow's milk. In addition the proportion of caseinogen in the protein is much higher in the case of cows' milk.

There is some variation in composition during the course of lactation. The milk secreted in the first few days after delivery, known as colostrum, is more yellowish in colour, and contains more protein than does later milk, averaging about 2·5 per cent. in human milk and sometimes reaching 4 to 5 per cent. During the remainder of lactation, while there is not much alteration in fat and lactose content, there is a steady fall in protein content, which towards the end of the first month averages about 1·7 per cent. as compared with an average of 1·1 per cent. at the end of nine months.

Human milk is sometimes examined in cases in which the child is not gaining weight satisfactorily, but in the great majority of cases is found to be well within the normal range in composition. Cows' milk may be tested occasionally if there is reason to suspect it is of low quality.

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*CHAPTER XXVI*

# STONES

THE biochemist is sometimes asked to investigate the nature of stones, concretions, or calculi, as they are variously known, which may be removed or excreted from the body. Stones from the urinary tract are by far the most common, but others occasionally studied include gall stones, intestinal concretions, salivary and pancreatic calculi.

## URINARY CALCULI

These may be found in any part of the urinary tract. They vary very considerably in size from little more than a pin-head to the size of an egg. Generally speaking the largest stones are found in the bladder, whilst stones from the kidney and renal pelvis are smaller, in some cases small enough to pass along the ureters and to be passed in the urine. But very large stones can be found in the kidneys, particularly in hydronephrosis.

Urinary calculi are almost always composed of substances normally excreted in the urine, together with a certain amount of protein material, including blood proteins. On rare occasions foreign bodies may be found and may form the nucleus of a stone.

It is possible to divide urinary calculi into three groups.

1. Simple calculi, which on examination are found to contain only a single urinary constituent.

2. Mixed calculi, which contain two or more substances present in the urine.

3. Foreign body calculi, composed either wholly or in part of some substance introduced into the body from outside.

Foreign body calculi are rare and of the first two groups mixed stones are the rule. It is unusual to find a stone composed of only one urinary constituent, though it is often found that one of the substances present is in marked preponderance.

Substances found in calculi include uric acid, urates, calcium oxalate, alkaline earth phosphates (calcium and magnesium), triple phosphate (magnesium ammonium phosphate), calcium carbonate, cystine, xanthine. Cholesterol and indigo calculi have been reported but are extremely rare. Fibrin and urostealith stones also occur only very infrequently. Well over 90 per cent. of stones contain uric acid, urates, oxalate, or phosphates, the two latter being most commonly found.

**Uric acid** stones are almost always coloured varying shades of brown. They are usually fairly hard and often have a smooth surface though sometimes they may be rather rough. Stones consisting entirely or chiefly of uric acid are not very common, but

it has been stated that uric acid forms the nucleus of stones in quite a high proportion of cases. This must be regarded as doubtful.

**Calcium oxalate** is found in a majority of calculi usually mixed with a greater or less amount of phosphates. Oxalate stones are among the hardest found and so are not easily crushed. The surface of small oxalate stones is often rough and bloodstained. Small stones from the renal pelvis often contain a considerable amount of oxalate, commonly from 65 to 80 per cent., mixed with a small quantity of phosphates and some organic matter.

**Phosphate calculi** are usually much softer than those mentioned above. They may contain either triple phosphate or calcium phosphate. Phosphates are most easily deposited when the urine is alkaline. Triple phosphate is usually found when there is infection of the urinary tract when urea is converted into ammonium carbonate. For this reason large bladder stones are usually mainly composed of phosphates.

**Calcium Carbonate.** Calculi consisting mainly of calcium carbonate are very rare in man but small amounts of this substance can be detected in a number of calculi. Stones containing carbonate tend to be rather hard.

**Cystine Stones.** These are also rare. They are usually fairly small, and are smooth and waxy in appearance and to the touch. In colour they are brownish-yellow or light green and occur in cases of cystinuria (see p. 96).

**Xanthine stones** are even rarer than cystine. They are yellowish-brown in colour being rather similar to uric acid calculi. They sometimes contain uric acid.

**Fibrin** calculi may form from blood clots. They are rare but may form the nucleus of other stones.

**Urostealith** calculi have been reported found in the bladder. They are soft and contain a large amount of fat. They are extremely rare.

**Foreign Bodies.** A remarkable variety of objects have been removed from the bladder or found as the centre of a calculus there. Pieces of catheter may be left in the bladder.

### Factors causing the Formation of Stones

It must be acknowledged that little is known in this connection. Certain conditions are recognized as being favourable for stone formation but do not go far to explain the presence of the majority of stones. It is known that stagnation of urine, particularly if there is also infection present, may lead to stone formation, for example, in hydronephrosis, enlarged prostate, and in bladder infections. Hyperparathyroidism and hypervitaminosis D with excessive mobilization and deposition of calcium are also known to be accompanied by formation of calculi, but they are exceedingly rare conditions. A defective diet, particularly in regard to vitamin A, has also been held to play a part, animal experiments having shown increased stone formation in such cases.

Substances such as calcium oxalate and uric acid are present in urine in supersaturated state and are thought to be kept in solution

by means of colloidal substances. So it is easy to see that conditions may arise leading to the formation of crystals. But for stone formation there must be some nucleus on to which these crystals can be deposited and then continue to grow. It is not a subject on which it is useful to speculate here.

### Chemical Examination of Urinary Calculi

The stone is best first washed with water and then dried in an incubator. Since most stones are mixtures and may consist of several layers, including a nucleus, it is desirable whenever possible, to separate these out and examine them separately. So the stone should be cut into two halves and examined for the presence of such layers and nucleus. However, while these may have a different composition it is sometimes found that layers differing in appearance have a similar composition. With small stones it is often not possible to examine in this way. These are powdered and examined.

**Reagents.** 1. Dilute hydrochloric acid.

2. Concentrated nitric acid.

3. Ammonium molybdate solution, freshly prepared by dissolving a few crystals in water.

4. Potassium hydroxide, 10 per cent. solution.

5. Nessler's reagent (see p. 104).

6. Dilute ammonia solution.

7. Concentrated ammonia solution.

8. Acetic acid.

9. Alcohol.

10. Ether.

**Technique.** The following order of investigation is recommended. Since some stones are quite small it is necessary to become accustomed to working with very small quantities of material for each test.

1. **Heat a small amount of the powder in a small crucible,** or better, on a **small platinum lid** or foil. Since most calculi contain at least a small amount of organic matter there is always a little charring, but if the stone is almost entirely inorganic this will be small in amount and a greyish powder will be left. If the stone is organic as in uric acid and cystine stones it will burn away entirely or leave only a trace of ash. It is generally clear which is the case but with some mixed stones containing both inorganic and organic material it may not always be possible to recognize the presence of relatively small proportions of substances such as uric acid.

Watch carefully *for the presence of a flame.* Uric acid, ammonium urate and xanthine burn away without producing a flame, but cystine gives a pale blue flame with a rather sharp smell, while fibrin and urostealith give yellow flames, the former with a smell of burnt feathers.

A. *If the substance burns away completely* it contains only organic matter. Oxalates, phosphates and carbonates are absent. Proceed as follows :

2. **Test for Uric Acid and Ammonium Urate by the Murexide Test.**

Add 2 or 3 drops of concentrated nitric acid to a small amount of the substance in a small evaporating dish or crucible and evaporate to dryness by heating on a water bath or very carefully over a small flame. The test is positive if a red or yellow residue is obtained which after being allowed to cool changes to a purplish red on addition of a drop of dilute ammonium hydroxide.

Xanthine does not give a murexide test. It dissolves in nitric acid leaving a yellow residue which on addition of alkali changes to orange and to red on warming.

3. **Test for cystine** if the murexide test is negative. This would probably be suspected by the behaviour of the calculus on heating. Dissolve some of the calculus in concentrated ammonia and allow this to evaporate off spontaneously on a watch glass. Hexagonal crystals of cystine will be deposited if it is present and should be examined microscopically. Cystine crystals are insoluble in acetic acid and in water, alcohol, ether and acetone but dissolve in dilute mineral acids, unlike uric acid crystals, which do not.

4. **Urostealith** in addition to burning with a yellowish flame with a resinous odour, is distinguished by its solubility in alcohol and ether.

5. **Fibrin** is insoluble in alcohol and ether but dissolves in hot potassium hydroxide solution from which it is precipitated by acetic acid, hydrogen sulphide being liberated.

B. *If a deposit remains after heating on platinum*, inorganic material is present. Test as follows :

6. Add a little dilute hydrochloric acid to a small portion of the stone. An effervescence shows the presence of *carbonate*.

7. After allowing to cool, add a little dilute hydrochloric acid to the ash remaining after heating on platinum, An effervescence now, in the absence of one before heating shows the presence of *oxalate*. To confirm, treat a small amount of the powder with warm dilute hydrochloric acid and filter. Only phosphates, cystine and oxalates dissolve. Make alkaline with ammonia. Oxalates and phosphates will be precipitated. The former are insoluble, the latter soluble, in acetic acid.

8. Dissolve a little of the powdered stone in a few ml. of concentrated nitric acid and then add an equal volume of ammonium molybdate solution. Heat to boiling. If *phosphates* are present, a yellow precipitate of ammonium phosphomolybdate is obtained.

9. Heat a little of the powder with 10 per cent. potassium hydroxide solution. *Evolution of ammonia* shows the presence of either *triple phosphate* or *ammonium urate*. Nessler's reagent may be used in testing for ammonia, bearing in mind its great sensitivity.

10. Phosphates, if present when tests for ammonia are negative, are those of calcium or magnesium. If desired the presence of these elements can be confirmed by the ordinary methods of inorganic analysis.

**Note well.** The fact that a deposit remains after heating does not exclude the presence of some organic substances, particularly uric

acid and ammonium urate. These should be tested for by the tests given above.

Although as a rule a rough idea can be obtained of the relative amounts of the different constituents present in a mixed stone, it may sometimes be useful to determine the amounts present, for example, in a mixed phosphate oxalate stone. Weigh out about 100 mg., dissolve in sulphuric acid, warm, and titrate with N/10 permanganate. One ml. of this is equivalent to 2 mg. of calcium or 6·4 mg. of calcium oxalate. Phosphate can be estimated using the method given under plasma inorganic phosphorus, dissolving a little of the powdered stone in the trichloracetic acid. Phosphorus forms about 23 per cent. whether the phosphate is triple phosphate ($NH_4MgPO_4$) or calcium hydrogen phosphate ($CaHPO_4$).

Determination of the chemical composition is only of limited value, but may help in connection with the institution of dietary measures, more particularly in the case of oxalate stones and uric acid stones. It will also reveal the presence of cystine when such stones are present, though this should usually have been discovered in the urine deposit.

## GALL STONES

Examination of gall stones is not often required, but it may occasionally be necessary to decide whether a fæcal concretion is or is not a gall stone. Examination of gall stones is easy. The substances found in them are cholesterol, bile pigments, calcium phosphate and calcium carbonate.

Most of the stones found in human beings consist mainly of *cholesterol* mixed with small amounts of pigments, calcium phosphate and protein material. They vary widely in size and colour, the latter depending mainly on the amount of pigment present. If they contain little or no pigment, they may be very light coloured even almost white ; otherwise they can be all shades from white to quite dark. Stones composed of *bile pigments* occur much less frequently. They are usually small and dark coloured. The pigment is mainly bilirubin present as calcium bilirubinate, but biliverdin and bilifuscin may be present. Small amounts of cholesterol may or may not be found. Traces of iron have been detected. Stones composed almost entirely of *inorganic material* are rare. They contain mainly calcium carbonate and calcium phosphate, sometimes with admixture of small amounts of pigments.

### Examination of Gall Stones

Wash with water, dry, and examine as follows :

1. Extract with ether. Powder the stone and heat some with successive small portions of ether in a test tube by inserting the tube in some warm water. Filter, evaporate off the ether, dissolve the residue in 95 per cent. alcohol and allow to crystallize. Typical cholesterol crystals with the notched corners (Fig. 28) are only

obtained if the crystallization is done from alcohol. The Liebermann-Burchardt reaction can be carried out. Dissolve a little of the residue obtained on evaporating off the ether, in chloroform and add a little of a mixture of acetic anhydride and sulphuric acid (in the proportion of 10 ml. to 0·1 ml.). A dark green colour develops rapidly.

2. Treat the residue remaining after ether extraction, with dilute hydrochloric acid (25 per cent.). This dissolves the inorganic salts present. Filter and test the filtrate for phosphates and for calcium. Make some of the solution alkaline with ammonia and add acetic acid and ammonium oxalate solution. If calcium is present a precipitate of calcium oxalate is formed. Traces of iron, if present, will also be in this filtrate.

3. Test the precipitate remaining after treatment with hydrochloric acid. for bile pigments. Wash the material remaining on the filter paper with water, dry the paper and extract with warm chloroform. Examine the chloroform extract for bilirubin by means of the diazo-reagent used in the Van den Bergh test. Alternatively, Hunter's diazo test can be carried out.

## PANCREATIC CALCULI

Pancreatic calculi are usually found either at operation or post mortem, but only occur rarely. They are small in size, have a rough appearance and are mostly composed of inorganic material, mainly calcium carbonate and calcium phosphate, but there may be traces of magnesium salts. The presence of oxalates has been reported. Small amounts of protein and fats may be present.

Investigation is simple. Treat with dilute hydrochloric acid. Effervescence shows the presence of carbonates, whilst phosphates and oxalates are dissolved. Filter. Make the filtrate alkaline with ammonia. Phosphates and oxalates are precipitated. Treat the precipitate with acetic acid. Phosphates dissolve but oxalates do not. Dissolve a little of the stone in nitric acid, add ammonium molybdate solution and warm. A yellow precipitate is given by phosphates. Add sodium phosphate solution to some of the ammoniacal solution above. A precipitate shows the presence of magnesium.

## SALIVARY CALCULI

Salivary calculi are occasionally submitted for examination. Like pancreatic calculi they are most commonly composed of calcium phosphate and/or calcium carbonate, with a small amount of organic matter. They are examined in the same way.

## FÆCAL CONCRETIONS

The biochemist is not often asked to examine fæcal concretions. Fæces may sometimes require to be examined for the presence of

*gall stones* if it is suspected that one has been passed. In addition to gall stones, which are the concretions most frequently found, several types of fæcal concretion have been recognized. Cammidge (1914) described the following :

**Enteroliths,** which consist mainly of phosphates deposited round a nucleus which is often a foreign body, such as a fruit stone, or pips. Some organic material and fæcal pigment are present. Formed in the large intestine, these are rare in man. Small enteroliths may also be formed by the deposition of phosphates on undigested vegetable material.

**Coproliths** are almost entirely organic, containing hardened fæcal material, formed from stationary fæcal matter in the colon.

**Drug Calculi.** Small stones may be formed from drugs which are insoluble or only slightly soluble, for example, magnesia, barium sulphate.

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## *CHAPTER XXVII*

# URINE AND FÆCAL PIGMENTS

## URINARY PIGMENTS

The main pigment present in normal urine, that which gives it its normal yellow colour is *urochrome*, the composition of which is not known and which does not give any absorption bands on spectroscopic examination. It can be adsorbed on to charcoal, and after drying this, extracted from it by alcohol. On evaporating off the alcohol it is obtained as a brown deposit. Traces of a number of other substances may also contribute to the colour of normal urine. These include uroerythrin, urobilin, uroporphyrin and coproporphyrin.

*Uroerythrin* is seen most characteristically as the pink pigment present in uric acid and urate deposits, with which it comes down. It is soluble in alcohol, ether, and chloroform, and can be extracted by means of amyl alcohol, in which its spectrum can be seen (see Fig. 68). Alkalies change its colour to green.

Whilst traces of *uroporphyrin* and *coproporphyrin* may be present they do not normally occur in sufficient quantity to be detected by spectroscopic examination or by ordinary tests.

*Urobilinogen* in urine becomes oxidized on standing, to *urobilin*, but the small amount normally present contributes little if anything to the colour of normal urine.

There is also said to be present in normal urine the colourless chromogen of *urorosein*. This substance, which appears to be a derivative of indole and to be formed in the intestine, can be converted into urorosein by the action of strong mineral acids. Like uroerythrin it can be extracted from urine by amyl alcohol. Its spectrum, shown in Fig. 68, has a band in the green.

Appreciable variations in the colour of urine occur according to whether it is dilute or concentrated. A very dilute urine may be almost colourless whilst a concentrated one may be deep yellowish-brown. Certain diseases lead to the production of dilute or concentrated urines. Thus, whilst *dilute urines* may be excreted as a result of heavy consumption of water, they are also produced in diabetes mellitus (see p. 72), in chronic nephritis, and in diabetes insipidus. In the case of diabetes mellitus the specific gravity is high, in the others low. *Concentrated urines* are excreted when the intake of water is low and when there is considerable sweating. This may be the case, for example, in fevers, and in conditions in which there is some circulatory defect, and it may be found in those diseases in which it is difficult to get the patient to take enough water. In many of these cases the amount of urinary pigments excreted daily is normal, but as a result of changes in the volume of water excreted, the concentration varies widely.

The amount of *urochrome* excreted daily appears to be independent of the diet and to be fairly constant in any particular person. It has been found by some workers to vary with the basal metabolism being increased in hyperthyroidism, and since the rate of metabolism is increased in fevers, the amount of uroehrome is increased, thus adding to the dark colour of the eoncentrated urine which is being excreted.

Increase of other pigments which are only present in small quantities in normal urines, is also found in disease. In particular, a marked increase in urobilin may be present in chronic liver disease, and in hæmolytic anæmias so that the urine often has a characteristic brown colour. In acute infective and toxic hepatitis, urobilinogen is present together with bilirubin, but the colour of the latter predominates.

Increased amounts of uroerythrin appear to be excreted in a number of pathological conditions and contribute to the reddish or deep orange tints then observed.

Urines which contain an increased amount of indican (p. 328) are often deeper brown in colour. If such urines become ammoniacal on standing some indigo may be formed. This can be extracted into chloroform which it colours blue to purple.

## Abnormal Pigments

Of the pathological pigments the greenish-yellow or brown of *bilirubin* is well known and its occurrenee in urine has been discussed (p. 232). The blood pigments which may be found in urine have also been considered (p. 394), the colour ranging from the red of *oxyhæmoglobin* to the brownish-black of *methæmoglobin* with all shades in between due to mixtures of these. The presence of abnormal amounts of *porphyrins* has also been discussed (p. 408). The port wine colour which may result is often quite typical.

Of the other pathological pigments, two merit detailed consideration, though they only occur rarely. These are melanin and homogentisic acid.

## Melanin in Urine

This type of pigment, never found in normal urine, occurs in patients with melanotic tumours, usually when there are secondaries in the liver. The eye is a common site for such a tumour which may be removed and the secondaries, which are very slow growing, may not become apparent until years later. Freshly passed urines do not contain the pigment, but a colourless precursor, melanogen, which is oxidized to melanin when the urine is allowed to stand in contact with air. Melanin is dark brown or black. So on standing, the urine darkens, often from the surface downwards, a process which usually occurs slowly, taking as long as twenty-four hours or more to become definitely noticeable. The tests used are for melanogen and so should be done on freshly passed urine.

**Ferric Chloride Test.** This is based on the oxidation by the ferric chloride of melanogen to melanin.

**Technique.** Add 10 per cent. ferric chloride drop by drop to 5 ml. of urine. The melanin may be carried down on the precipitate of ferric phosphate which forms thus giving a greyish or blackish precipitate. As more ferric chloride is added, the precipitate dissolves, leaving a slightly turbid fluid coloured varying shades from brown to black according to the amount of melanin present.

Harrison (1947) uses a 10 per cent. solution of ferric chloride in 10 per cent. hydrochloric acid. The acid prevents the formation of a precipitate so that the colour change only is observed. It is claimed that the colour thus obtained is often darker than that given by 10 per cent. ferric chloride in water so that smaller amounts of melanogen can be detected.

**Nitroprusside Test of Thormahlen. Reagents.** 1. Sodium nitroprusside solution. Prepare freshly before use by dissolving a few crystals in a few ml. of water.

2. Thirty-three per cent. (v/v) acetic acid.

3. Forty per cent. sodium hydroxide.

**Technique.** Add 3 or 4 drops of the nitroprusside solution to about 5 ml. of urine and make strongly alkaline with about 0·5 ml. of the sodium hydroxide. Shake well to mix and make acid by adding a few ml. of acetic acid. The presence of melanogen is shown by the development of a blue to blue-black colour. The actual colour seen depends on the colour of the original urine. If this was deep yellow the colour seen will be dark green. The less pigmented the urine, the bluer the colour produced.

This test is similar to the nitroprusside test for acetone and acetoacetic acid, when a reddish-purple colour is obtained. Creatinine gives a brown colour.

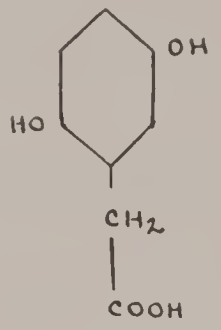
It is advisable to become familiar with the sort of colour which normal urines may give. This may be slightly brownish with a green tinge. Contrast a suspected positive with a normal control.

**Other Tests.** Several other tests for melanogen have been described but are not so satisfactory and are not likely to be positive if the above are negative. These include the *nitric acid test*, in which a few drops of concentrated nitric acid are added to a few ml. of urine, a brown to black colour being given by melanogen ; the *bromine test*, in which bromine water is added to urine, the precipitate which forms darkening through grey to black if melanogen is present ; the *silver nitrate test*, in which 0·5 ml. of urine is added to 5 ml. of 3 per cent. silver nitrate and 2 per cent. ammonia added until most of the precipitate has redissolved, when if melanogen is present the mixture slowly darkens to brown and finally black. Keep away from sunlight.

Melanin can be precipitated from urine by methyl alcohol after oxidation of the melanogen present. Collect a twenty-four-hour specimen and evaporate to about a quarter of the original volume. Add 1 gram of potassium persulphate per 100 ml. and after two hours add an equal volume of methyl alcohol. Filter and wash in turn with water, methyl alcohol and ether.

### Homogentisic Acid in Urine, Alkaptonuria

In this condition the urine also darkens on standing. This is due to the conversion of homogentisic acid, the formula of which is shown, into substances of unknown composition which are coloured brown or black. This colour also appears in the surface layer. It develops much more quickly in alkaline than in acid urine and comes




Homogentisic acid

up within a few seconds when the urine is made alkaline with sodium or potassium hydroxide or with ammonia. This is a difference from melanogen in urine on which the addition of alkali does not speed up the oxidation. With melanogen in urine it is an acid medium which favours the development of the colour.

Metabolism of tyrosine and phenylalanine is defective in this condition so that instead of these substances being completely metabolized, homogentisic acid is formed and excreted in the urine. The condition may be first noticed in early infancy when the napkins are seen to be stained. In later life cartilages become blackened and may give a bluish tinge to the hollows of the ears and brown coloration to the conjunctivæ.

As stated earlier (p. 68) urines containing homogentisic acid reduce alkaline copper solutions. Thus on boiling with Benedict's solution a greenish-brown colour first results, giving a brownish precipitate which when allowed to settle is seen to be similar to the cuprous oxide formed with other reducing substances. This again contrasts with the behaviour of urine containing melanogen which may give a greenish-black precipitate if an appreciable amount is present but otherwise shows little or no change.

Other tests use the behaviour towards ammoniacal silver nitrate and with ferric chloride. When a few drops of 10 per cent. ammonia are added to a mixture of 0·5 ml. of urine and 5 ml. of 3 per cent. silver nitrate solution a black colour is produced. Indeed in alkaptonuria the black colour often develops without the addition of ammonia. The mixture must not be exposed to direct sunlight. On adding a few drops of 10 per cent. ferric chloride to a few ml. of urine, in addition to the formation of ferric phosphate a transient blue or green colour occurs in alkaptonuria. This may often be seen better when a more dilute ferric chloride solution is used. With melanogen ferric chloride gives a brown to black colour.

The nitroprusside test and the reactions with nitric acid and bromine described for melanin are not given by the urine in alkaptonuria.

It should be noted that a green colour with ferric chloride solution, which fades after only a few minutes, is given by the urine in *phenylketonuria*. The urine in this differs from alkaptonuria both in not darkening in air and in not reducing either alkaline copper solutions or silver nitrate.

Homogentisic acid can be estimated by using the colour produced with ammonium molybdate and potassium dihydrogen phosphate using as standard a solution of hydroquinone similarly treated. Take 1 or 2 ml. of urine, dilute to 15 ml. with water, add 2 ml. 5 per cent. ammonium molybdate in 5 N sulphuric acid and 2 ml. of 1 per cent. potassium dihydrogen phosphate and dilute to 25 ml. Treat a hydroquinone standard containing 1 mg. per ml. in the same way. One mg. of hydroquinone equals 0·79 mg. of homogentisic acid. If albumin is present in the urine it must be removed before the estimation is done.

### Presence of Drugs, Dyes and other Ingested Substances

In addition abnormally coloured urines may be due to substances taken by the person. In the first place pigments present in *foods* are sometimes excreted in the urine. Thus *anthocyanins* in fruits and vegetables may give coloured urines. One of the most notable of these is that present in beetroot which gives a reddish-pink colour superficially resembling hæmoglobinuria, though the colour is actually rather pinker and fairly easily distinguished when the worker is familiar with it. This colour acts as an indicator, being yellow with alkalies and pinkish-red with acids. Large quantities of rhubarb may give a somewhat similar colour. Fruits such as blackberries and bilberries may also give coloured urines in some persons. These pigments are not extracted by amyl alcohol.

Dyes present in such articles as *sweets* may appear in the urine. Thus *eosin* may give a characteristic appearance to urine, showing a greenish fluorescence. Amyl alcohol extracts the dye which on spectroscopic examination shows a band in the green. Enquiry may reveal that such substances have been taken, but in other cases the behaviour of the pigment, with failure to demonstrate any pigment pathologically significant, leads to the conclusion that such must be the source of the colouring matter.

The colour of the urine is important in *phenol and lysol poisoning*, when it is brownish or black, though it may also sometimes have a greenish tinge in phenol poisoning. Phenol is excreted to some extent unchanged, in part conjugated with glucuronic and sulphuric acids, but the greater part is found as hydroquinone and pyrocatechol and their oxidation products. It is the amount of these which mainly determines the colour. Although tests may be carried out on the urine directly, it is better to acidify the urine with sulphuric acid and distil. Tests are then done on the distillate. Phenols give a white or yellow precipitate with bromine water due to the formation of

tribromphenol and give a colour, blue with phenol and greenish with cresols, with 10 per cent. ferric chloride solution. Such urines may give some reduction with alkaline copper solutions since the phenol may be excreted as glucuronates.

Urines may also be abnormally coloured by drugs taken and occasionally by substances used in certain tests. As regards the latter *phenol red* is used in a test of renal function. It is red in alkaline and yellow in acid solution. *Congo red* used in testing for amyloid disease may be excreted in some of these cases and colour the urine red. *Indigo carmine* has also been used in renal disease colouring the urine greenish-blue. Boiling the urine with a few drops of 40 per cent. sodium hydroxide removes the colour.

Amongst drugs which should be noted in this respect are the following. *Methylene blue* gives the urine a green or greenish-blue colour, is extracted by chloroform and by amyl alcohol, and has an absorption band in the red. Harrison (1947) has described a test for methylene blue in which it is reduced to its leuco base. This, on cooling and shaking with air, is reoxidized to the dye. A little glucose is added to a few ml. of the urine, followed by 1 to 3 drops of 40 per cent. sodium hydroxide (not sufficient to remove the green colour). The mixture is then boiled for a few minutes. The colour disappears but can be brought back by cooling and shaking in air. *Indigo*, which has a similar spectrum to methylene blue and is also extracted by chloroform, does not give the above test and further, when its solution in water is evaporated to dryness the residue on being gently heated gives a purple sublimate in contrast to the fainter green one given by methylene blue. In addition to its use as a drug and in certain proprietary medicines, methylene blue may also be present as a colouring matter in sweets. It is the commonest cause of blue-green urines.

*Phenylhydrazine* which has been used in treating polycythæmia may produce brown urines. These may be due to the formation of aromatic oxidation products which can be extracted from the acidified urine by amyl alcohol. Phenylhydrazine may also bring about the formation of methæmoglobin and thus cause methæmoglobinuria.

*Phenolphthalein* is contained in some purgatives and may be excreted unchanged in the urine. It is rare for urines to be alkaline enough to give a red colour with this indicator, but they may become so on standing.

*Amidopyrine* also gives urines coloured pink to red. It is extracted by amyl alcohol and the colour changes to yellow when the urine is made markedly acid or alkaline.

*Pyridium* may also colour the urine orange to orange red. The colour becomes redder on adding hydrochloric acid, but is not altered appreciably by caustic soda. The dye is extracted by amyl alcohol.

### Examination of Pigmented Urines

In examining urines containing abnormal pigments direct observation gives much information to those familiar with such urines, but

a few tests are of outstanding value. These include spectroscopic examination of the urine and of its amyl alcohol extract ; the behaviour of the colour to acid and alkali ; and the colour given with ferric chloride solution. If none of the pigments known to be present in pathological conditions is detected, the colour is almost certainly due to some foreign substances introduced into the body usually in the food or as drugs, though in the case of young children chewing coloured articles may be responsible. Enquiry will often yield information as to whether this has occurred.

## FÆCAL PIGMENTS

The normal colour of fæces is much influenced by the amount of urobilin (stercobilin) present, but it also depends to a considerable extent on the kind of food taken. As regards urobilin, there is further production of this on standing so that fæces then darken in colour, particularly on the surface. A largely vegetable diet gives light-coloured stools whilst one rich in meats gives darker, brown ones. Undigested portions of some vegetables may colour the fæces, reddish-brown in the case of carrots, greenish in the case of vegetables such as spinach which are rich in chlorophyll. Fruits, too, may colour the stools, for example, bilberries which give them a greyish-purple appearance. On a milk diet the stools are pale yellow or orange.

Normal adult fæces do not contain bilirubin or biliverdin, which is pathological and indicates the rapid passage of the intestinal contents. The stools are then coloured from brownish-yellow to green. Green stools in very young infants may be due to the presence of biliverdin. Meconium may be green.

Clay-coloured stools—acholic stools—found in obstructive jaundice are associated with the absence of bile pigments from the intestine. The presence of a considerable amount of unabsorbed fats may also help to produce such stools. Whitish or cream-coloured fæces have been said to occur in cases of pancreatic disease in which there was no jaundice.

When blood is present, fæces may be coloured all shades from brownish-red to black. The darker colours occur in cases where the hæmorrhage is high up in the alimentary tract, for example, in gastric and duodenal ulcer. Blood from the rectum and anus may show as streaks of fresh blood on the surface of the stool.

Drugs may influence the colour of the fæces. Barium sulphate given as a barium meal gives whitish-coloured stools ; calomel is said to produce a greenish tint ; iron salts used in treating anæmia give dark, almost black fæces, as does bismuth subnitrate in which case the suboxide is formed.

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*CHAPTER XXVIII*

# DRUGS AND POISONS

In this chapter methods will be given for the determination in body fluids of a number of miscellaneous substances mostly drugs and poisons. It is sometimes useful to know the blood level of a drug which is being given, either to ensure that the optimum blood concentration has been achieved, or to ensure that overdosage is not occurring. There are also a few poisons, the determination of which may reasonably be considered to be within the scope of the hospital biochemist. Otherwise work dealing with poisons is better left to the forensic laboratory.

## ALCOHOL

### Determination in Blood and Urine

The method most commonly used is to distil the alcohol into a mixture of potassium dichromate and sulphuric acid. Some dichromate is used in oxidizing the alcohol. The dichromate remaining is determined iodometrically by adding potassium iodide and titrating the iodine liberated, with thiosulphate.

**Reagents.** 1. N/10 potassium dichromate.

2. Concentrated sulphuric acid.

3. Potassium iodide, 5 per cent. solution.

4. N/10 sodium thiosulphate.

**Technique.** Connect up three 100 ml. cylinders or 8 × 1 inch test tubes, so that air can be drawn through. These are joined in the same way as the tubes in the urease—aeration method for blood urea (Fig. 30), the inlet tube reaching to the bottom while the outlet tube only just passes through the cork. Place 5 to 10 ml. of concentrated sulphuric acid in the first tube, 2 ml. of blood or urine in the second, and a mixture of 10 ml. N/10 dichromate and 10 ml. concentrated sulphuric acid in the third. Partially immerse the second and third tubes in a water bath at 80° C. and draw air through until the specimen is evaporated. Up to an hour may be required. Trouble may be experienced from frothing. A circular piece of fine iron wire gauze placed about halfway up the tube can be used to break the froth. When aeration is finished cool the third tube and transfer the contents with washing to a flask, making to a volume of 300 to 400 ml., add 5 ml. of 5 per cent. potassium iodide and titrate with N/10 thiosulphate. Titrate 10 ml. of the N/10 dichromate in the same way. This should require 10 ml.

**Calculation.** Since 1 ml. of N/10 dichromate is equivalent to 1·15 mg. of alcohol.

Mg. alcohol per 100 ml. of specimen

$$= (\text{Titration of standard} - \text{titration of test}) \times 1{\cdot}15 \times \frac{100}{\text{Ml. blood or urine used}}$$

$$= (10 - \text{titration of test}) \times 57{\cdot}5, \text{ if 2 ml. is used.}$$

**Use of Conway Units.** The Conway micro-diffusion unit is convenient to use. Made of Pyrex glass, this is shaped like a small Petri dish and is divided into an inner and outer compartment by a circular inner glass wall the height of which is about half that of the outside wall. The procedure for alcohol is as follows (Conway, 1947). Pipette 0·5 ml. of 0·4 N potassium dichromate in 10 N sulphuric acid into the inner compartment. As fixative use a mixture prepared by melting 40 to 50 grams of paraffin wax (m.pt. about 40° C.) with 80 ml. pure liquid paraffin and cooling. Smear the lid with this ready for applying before running a suitable amount of blood or urine into the outer compartment. When this has been done seal with the lid immediately. A volume of specimen containing from 1·0 to 1·5 mg. of alcohol is most convenient. Use 0·25 ml. in severe intoxication, 0·5 ml. in moderate, 1·0 ml. in other cases. Move the units to spread the liquids over as great an area as possible. Stand overnight at room temperature or for six hours at 37° C. Then remove the lid, dilute the dichromate with 1 ml. of water, add 0·3 ml. of 3 M potassium iodide, stir well with a fine glass rod and titrate immediately with N/10 thiosulphate. Run this in quickly at first while stirring slowly, until a pale yellow-green colour is reached. Add a drop of 1 per cent. starch solution and complete the titration. Carry out a blank using water instead of the specimen under test.

Mg. alcohol in the volume of specimen actually used

$$= (\text{Titration of blank} - \text{titration of test}) \times 1{\cdot}152$$

For a full account of the various techniques using micro-diffusion see Conway (1947).

INTERPRETATION

There is very little alcohol in normal blood or urine. Values of blood alcohol up to 100 mg. per 100 ml. are found in subclinical states of intoxication. Above this some degree of intoxication may be said to be present. This is mild up to about 180 mg. per 100 ml. Some degree of confusion is usually present over 200 mg. per 100 ml. Intoxication is severe with stupor over 300 mg. per 100 ml. Values up to and a little over 500 mg. per 100 ml. may be found, coma being present above about 400. There is, however, a considerable personal variation at all levels. Nevertheless, on the average intoxication may be said to commence at 100 mg. per 100 ml. and become serious at 200 mg. per 100 ml. The concentration in the urine is usually from a third to a half higher than that in the blood.

## BARBITURATES

**Estimation of Barbiturates in Body Fluids** (Levy, 1940)

This method may be used for blood, urine, or cerebrospinal fluid. The fluid is dried on anhydrous sodium sulphate and the barbiturates

extracted with an ether-petroleum ether mixture in a Soxhlet apparatus. After evaporation of the solvent the residue is dissolved in chloroform and estimated colorimetrically, using the purple colour which is produced on the addition of cobalt acetate in methyl alcohol and isopropylamine.

**Reagents.** 1. Sodium dihydrogen phosphate.

2. Anhydrous sodium sulphate.

3. Ether-petroleum ether extraction mixture. Mix equal volumes of peroxide-free ether (prepare weekly by washing with ferrous sulphate solution, then with water, and distilling into a bottle containing clean copper wire) and petroleum ether (b.p. 30° to 40° C.).

4. Decolorizing mixture. Mix 3 parts of prepared charcoal (wash with peroxide free-ether containing a few drops of glacial acetic acid, and then with ether until free from acid) with one part of magnesium oxide.

5. Cobalt acetate. One per cent. solution in absolute methyl alcohol.

6. Isopropylamine. Five per cent. by volume in absolute methyl alcohol.

7. Standard solution of barbituric acid. Prepare solutions of diethyl barbituric acid (not the sodium salt) in chloroform. A stock solution containing 200 mg. per 100 ml. is convenient. From this prepare a series of standards by diluting with chloroform. These should contain 0, 20, 40, 60, 80, 100 mg. per 100 ml. Keep in a dark glass-stoppered bottle.

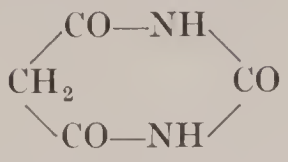
**Technique.** Mix 20 ml. urine (or as much blood, or cerebrospinal fluid as is available) in a mortar with 2 grams of sodium dihydrogen phosphate. Then add 40 grams of anhydrous sodium sulphate gradually in small portions with constant grinding. After thorough mixing place the mortar in a desiccator for thirty minutes or longer to allow complete drying to take place. Break up the dried mixture and place in the thimble of a Soxhlet extraction apparatus, and extract with ether-petroleum ether mixture for about three hours. Shake the extract with about 0·25 gram of the decolorizing mixture. Remove this by filtering directly into a distillation flask, washing in with more extraction mixture. Distil off the ether and petroleum ether and take up the residue in 10 ml. of chloroform. To 2 ml. of this chloroform solution add 0·1 ml. of cobalt acetate solution and 0·6 ml. of isopropylamine solution and compare with 2 ml. of the appropriate standard solution similarly treated. The colours can be read in the photoelectric calorimeter, using a yellow green filter or transmission at 540 millimierons.

**Calculation.** If the photoelectric colorimeter is used :

$$\text{Mg. barbituric acid per 100 ml. of specimen} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \text{Mg. barbituric acid per 100 ml. of standard} \times \frac{\text{Total volume of chloroform extract}}{\text{Volume of chloroform extract used for colour development}} \times \frac{100}{\text{Volume of specimen used}}$$

INTERPRETATION

Barbiturates are now widely used as sedatives and as anæstheties. They are derivatives of barbituric acid, malonyl urea :



The hydrogen atoms of the $CH_2$ group are substituted by various alkyl and aryl groups. Important examples are :

| | |
|---|---|
| Diethyl barbituric acid (barbitone, veronal) . . | M. Wt. 184 |
| Sodium diethyl barbiturate (barbitone soluble) . . | M. Wt. 206 |
| Isoamyl ethyl barbituric acid (amytal) . . . | M. Wt. 226 |
| and its sodium salt (sodium amytal, amytal soluble) | M. Wt. 248 |
| N-methyl cyclohexenyl methyl barbituric acid (evipan) | M. Wt. 236 |
| and its sodium salt (sodium evipan) . . . | M. Wt. 258 |
| Ethyl phenyl barbituric acid (phenobarbitone, luminal) | M. Wt. 232 |
| N-methyl ethyl phenyl barbituric acid (luminal) . | M. Wt. 246 |
| Sodium ethyl methylbutyl barbiturate (pentobarbital, nembutal) . . . . . | M. Wt. 248 |
| Diallyl barbituric acid (Dial) . . . . . | M. Wt. 208 |

Barbituric acids are soluble in ether to varying degrees and can be extracted by acidifying and shaking with ether or chloroform.

## BROMIDES

The recognition that taking excessive doses of bromides may lead to a state of intoxication with various abnormal mental manifestations led to the development of a simple method for determining serum bromide which provides a quick means of confirming that such a condition is present.

**Determination of Serum Bromide** (Barbour *et al.*, 1936)

After precipitating the proteins with trichloroacetic acid, the bromide in the filtrate is estimated by comparing the brown colour formed on adding gold chloride with standard sodium bromide solutions similarly treated.

**Reagents.** 1. Trichloracetic acid, 20 per cent. (w/v) solution.

2. Gold chloride ($AuCl_3$, $HCl$, $3H_2O$), 0·5 per cent. in water.

3. Stock solution of sodium bromide. Dissolve 145 mg. of sodium bromide in water and make to 100 ml.

**Technique.** Pipette 2 ml. of the serum into a test tube and add 4 ml. of distilled water, followed by 1·2 ml. of 20 per cent. trichloracetic acid. Shake well and filter through a 7 cm. No. 1 Whatman paper. To 4 ml. of the filtrate add 0·8 ml. of the 0·5 per cent. gold chloride solution. Compare the resulting colour with a set of standards prepared in similar sized test tubes. These may be prepared

| Mg. bromide as NaBr per 100 ml. serum | 0 | 25 | 50 | 75 | 100 | 125 | 150 | 175 | 200 | 225 | 250 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ml. stock standard | 0 | 0·25 | 0·5 | 0·75 | 1·0 | 1·25 | 1·5 | 1·75 | 2·0 | 2·25 | 2·5 |
| Ml. water | 5 | 4·75 | 4·5 | 4·25 | 4·0 | 3·75 | 3·5 | 3·25 | 3·0 | 2·75 | 2·5 |

Add 1 ml. of 20 per cent. trichloracetic acid and 1·2 ml. of 0·5 per cent. gold chloride solution.

These tubes can be sealed off and kept in the dark, being then used for comparison with the test when required. The tubes will keep for several months. A standard curve for the colorimeter can be prepared using a blue filter, transmission at 470 millimicrons ; or suitable standards such as the 100 and 200 mg. per 100 ml. may be used for the visual colorimeter.

**Calculation.** The figures given for the serum bromide above are corrected so as to allow for a small error which may occur because some bromide is adsorbed on to the protein precipitate.

**Note.** Iodides interfere with the test since they give a similar colour with gold chloride. Accordingly when a positive result is obtained it is advisable to check that the patient has not been taking iodides.

INTERPRETATION

There is normally only a very small amount of bromide in blood, not more than 3 mg. per 100 ml., which is not sufficient to show any colour with the above test. Symptoms of intoxication appear when the serum bromide, expressed as NaBr, is between 100 and 200 mg. per 100 ml. There are considerable personal variations. With increasing serum bromide the symptoms become increasingly severe until, with values of over 300, the patient becomes demented. There is replacement of chloride by bromide. When there is 50 per cent. replacement death is said to occur. This corresponds to a serum bromide over 400 mg. per cent. On stopping the drug and giving liberal quantities of fluid and sodium chloride the serum bromide falls steadily. The symptoms of intoxication disappear in two or three days and the serum bromide may become normal again in a fortnight.

## SALICYLATES

Large doses of salicylates have been used in the treatment of rheumatic disease and determinations of the amount in serum (or plasma) have been used to control such treatment. A rapid simple method for such estimation is thus of value.

**Determination of Serum Salicylates.** Method of Keller (1947)

The colour formed by reaction of salicylates with acidified ferric nitrate is used. Proteins are not precipitated.

**Reagents.** 1. Approximately 0·07 N nitric acid. Make 4·69 ml. of concentrated nitric acid (S.G. 1·42, 70·5 per cent. by weight) up to 1 litre with water. Extreme accuracy is not essential.

2. One per cent. ferric nitrate, $Fe(NO_3)_3$, $9H_2O$ in 0·07 N nitric acid.

3. Dilute ferric nitrate. Add 4 volumes of water to 5 volumes of the 1 per cent. solution.

4. Nitric acid, 0·038 N. Dilute 5 volumes of 0·07 N nitric acid with 4 volumes of water.

5. Stock salicylic acid or sodium salicylate solution. Dissolve 100 mg. of salicylic acid or 116 mg. of sodium salicylate in water and make up to 100 ml.

6. Standard solution for use. Dilute the stock solution 1 in 10 for use in preparing a standard curve for the photoelectric colorimeter.

**Technique.** In a suitable test tube add 9 ml. of the diluted ferric nitrate solution to 1 ml. of serum. In another tube, as blank, add 9 ml. of 0·038 N nitric acid to 1 ml. of serum. Read on the colorimeter, using a green filter, transmission at 520 millimicrons.

Prepare a standard curve as follows :

| Mg. salicylic acid per 100 ml. | 0 | 5 | 10 | 20 | 30 | 40 | 50 |
|---|---|---|---|---|---|---|---|
| Ml. standard salicylate solution | 0 | 0·5 | 1·0 | 2·0 | 3·0 | 4·0 | 5·0 |
| Ml. of water | 5·0 | 4·5 | 4·0 | 3·0 | 2·0 | 1·0 | 0 |
| Ml. 1 per cent. ferric nitrate. Add 5 ml. to each. | | | | | | | |

For visual colorimetry select a suitable standard, the 20 mg. per 100 ml. being the most useful.

**Calculation.** A certain amount of colour is obtained on adding ferric nitrate to sera from persons not taking salicylates. In preparing the standard curve an average value for this should be determined. To do this, pool samples of serum from about ten normal persons not taking salicylates, and repeat the above technique as given for the

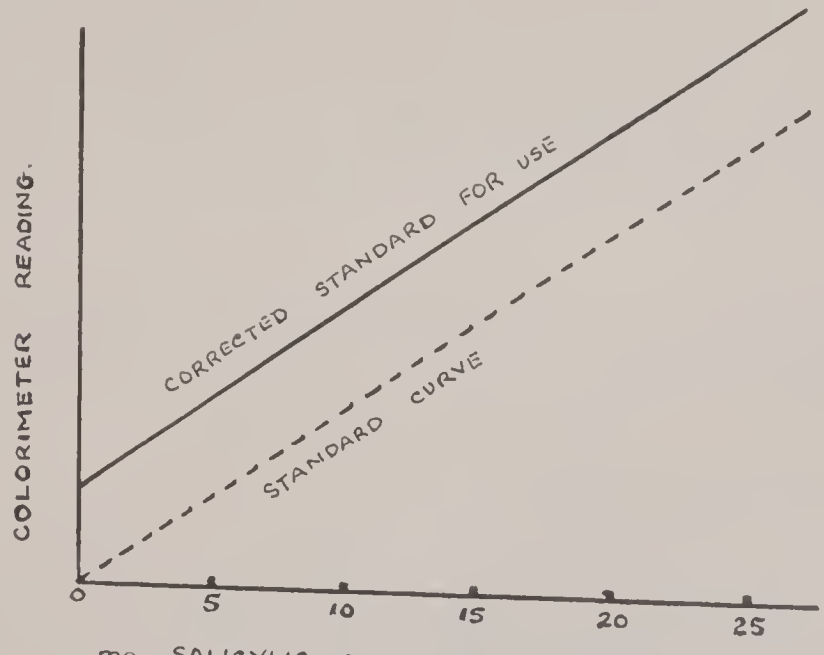



FIG. 70. Determination of Salicylates.

estimation. It is found that the colorimeter reading corresponds to about 4 to 5 mg. per 100 ml. of salicylic acid. Variations in any single case very rarely exceed 1·5 mg. per 100 ml. The colorimeter reading of this blank can be added to the reading of the standards to give a curve which then automatically corrects for the colour from these substances present in normal serum (Fig. 70). The possible error of ± 1·5 mg. per 100 ml. is not serious when compared with the levels of serum salicylate which should be reached in treatment.

**Notes.** 1. Serum is preferable. If done on plasma this should not contain more than 6 mg. of sodium oxalate or its equivalent per 10 ml.

2. 0·5 ml. serum may be used, particularly for high levels. Add 4·5 ml. of water and 5 ml. of diluted ferric nitrate. Put up a blank from 0·5 ml. serum, 4·5 ml. water and 5 ml. of 0·038 N nitric acid. Prepare a standard curve accordingly.

3. The test can be adapted for urine. Add 0·5 ml. water and 9 ml. of diluted ferric nitrate to 0·5 ml. urine. Prepare standards using pooled normal urines containing no salicylates. Large quantities of salicyluric acid in the urine invalidate the test except as an indication of the total salicyl output. (Brodie *et al.*, 1944.)

*Other Methods.* Use has also been made of the reaction of salicylates with the Folin-Ciocalteu phenol reagent to give a blue colour on addition of sodium hydroxide (Smith and Talbot, 1950). The technique is as follows : add 0·2 ml. of serum to 5·8 ml. distilled water. Then add 3 ml. of the Folin-Ciocalteu reagent, diluted 1 to 3 with distilled water, and centrifuge for five minutes. Pipette 4 ml. of the supernatant fluid and add 2 ml. of 1·5 N sodium hydroxide. Stand for three minutes and read in the photoelectric colorimeter using a red filter, or transmission at 670 millimicrons. Construct a calibration curve by treating appropriate solutions of salicylic acid or salicylates in water in the same way. This method gives a higher blank with sera from normal persons not taking salicylates than does the ferric nitrate method, the range being from 4·5 to 9·5 mg. per 100 ml. in terms of salicylic acid, the average being 7·5 mg. per cent. Uric acid as well as tyrosine and tryptophane react to give the colour.

INTERPRETATION

In treatment of rheumatism levels of about 35 mg. per 100 ml. for serum salicylic acid are desirable for effective treatment.

## *p*-AMINOSALICYLIC ACID

**Determination in Serum** (Smalley, 1949)

Proteins are precipitated with trichloracetic acid and the estimation made on the protein-free filtrate using the orange colour with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent).

**Reagents.** 1. Trichloracetic acid, 10 per cent. (w/v) solution.

2. N/4 sodium hydroxide.

3. Ehrlich's reagent. Dissolve 3 grams of *p*-dimethylaminobenzaldehyde in 7 ml. of sulphuric acid and make to a litre with water.

4. N sodium hydroxide.

5. N hydrochloric acid.

6. Stock solution of *p*-aminosalicylic acid. Dissolve 20 mg. in 100 ml. of 10 per cent. trichloracetic acid.

7. Standard solution of *p*-aminosalicylic acid for use. Dilute 1 ml. of stock solution to 20 ml. with 10 per cent. trichloracetic acid. One ml. then contains 0·01 mg. of *p*-aminosalicylic acid.

**Technique.** Mix 0·2 ml. of blood with 2·8 ml. of 10 per cent. trichloracetic acid in a centrifuge tube. Allow to stand for a few minutes. Centrifuge. To 1·5 ml. of the clear supernatant fluid add 2 ml. of N/4 sodium hydroxide and 1 ml. of the Ehrlich reagent and mix. An orange colour develops at once and is stable for several hours. Read in the photoelectric colorimeter using a blue filter, transmission at 470 millimicrons. A standard curve can be prepared as follows :

| Mg. *p*-aminosalicylic acid per 100 ml. | 0 | 2 | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|---|
| Ml. of standard containing 0·01 mg. per ml. | 0 | 0·2 | 0·4 | 0·6 | 0·8 | 1·0 |
| Ml. 10 per cent. trichloracetic acid | 1·5 | 1·3 | 1·1 | 0·9 | 0·7 | 0·5 |

Add 2 ml. N/4 sodium hydroxide and 1 ml. Ehrlich reagent.

**Method for Urine.** Dilute the urine 1 to 500 with distilled water, add 1·5 ml. N hydrochloric acid to 0·5 ml. of the diluted urine and place in boiling water bath for thirty minutes. Cool and make the volume to 2 ml. with water, add 1·5 ml. of N sodium hydroxide, 0·3 ml. of distilled water and 1 ml. of Ehrlich's reagent. Prepare a standard curve.

| Grams. *p*-aminosalicylic acid per 100 ml. urine containing | 0 | 0·2 | 0·4 | 0·8 | 1·2 | 1·6 |
|---|---|---|---|---|---|---|
| Ml. standard 0·02 mg. per ml. | 0 | 0·1 | 0·2 | 0·4 | 0·6 | 0·8 |
| Ml. 10 per cent. trichloracetic acid | 1·0 | 0·9 | 0·8 | 0·6 | 0·4 | 0·2 |

Add 1 ml. N hydrochloric acid to each, heat as above for thirty minutes in a boiling water bath. Cool, make to 2 ml., add 1·8 ml. N sodium hydroxide and 1·0 ml. Ehrlich's reagent.

INTERPRETATION

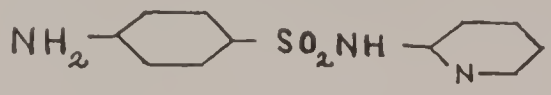
Blood levels required are in the range 4 to 8 mg. per 100 ml. The twenty-four hour excretion in the urine is 10 to 11 grams with patients taking 14 grams daily.

## SULPHONAMIDES

Since the introduction of sulphanilamide a wide range of substances derived from it by substitution of other groups in the amino groups in the molecule have been used, of which some are listed below.

*Sulphanilamide*, *p*-aminobenzene sulphonamide, $NH_2C_6H_4SO_2NH_2$, m.p. 164·5° to 166·5° C. Molecular weight 172.

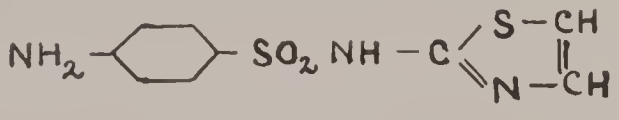
*Sulphapyridine*, 2 (*p*-aminobenzenesulphamido) pyridine, $NH_2C_6H_4SO_2NHC_5H_4N$,



Sulphapyridine

m.p. 191° to 193° C. Molecular weight 249.

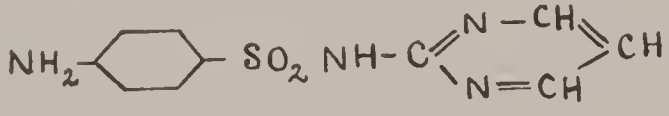
*Sulphathiazole*, 2 (*p*-aminobenzenesulphamido) thiazole,



Sulphathiazole

m.p. 200° to 203° C. Molecular weight 255.

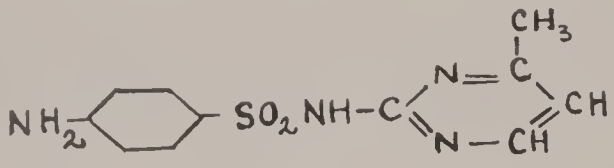
*Sulphadiazine*, 2 (*p*-aminobenzenesulphamido) pyrimidine,



Sulphadiazine

m.p. 252° to 256° C. Molecular weight 250.

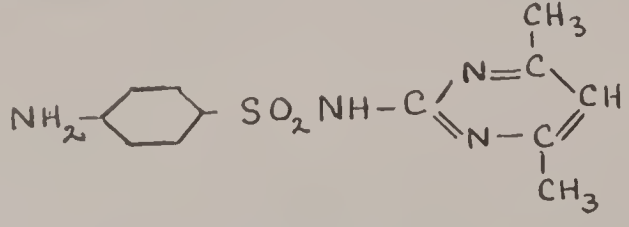
*Sulphamerazine*, sulphamethylpyrimidine, m.p. 235° to 238° C.



Sulphamerazine

Molecular weight 264.

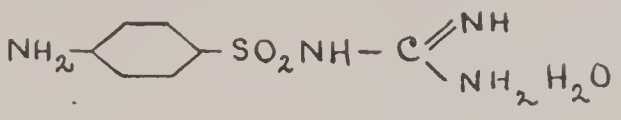
*Sulphamethazine*, sulphadimethylpyrimidine,



Sulphamethazine

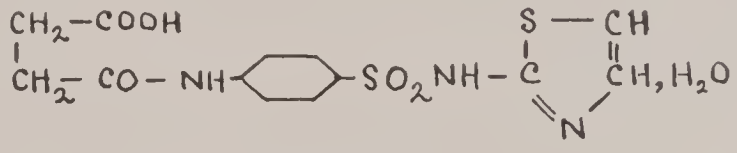
Molecular weight 278.

*Sulphaguanidine*, sulphanilylguanidine, usually occurs with one molecule of water of crystallization. M.p. 190° to 192·5° C. on the dried material. Molecular weight 232, anhydrous 214.



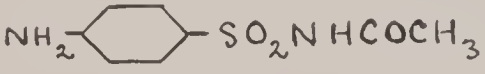
Sulphaguanidine

*Sulphasuccidine*, succinylsulphathiazole. Molecular weight 373.



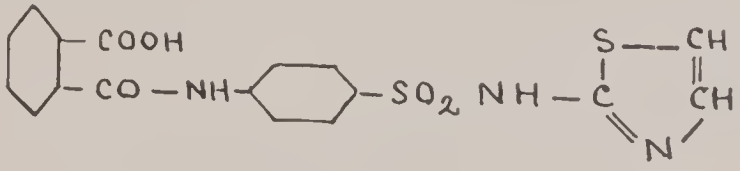
Sulphasuccidine

This also usually occurs with one molecule of water of crystallization.
*Sulphacetamide*, *p*-aminobenzenesulphonacetamide.



Sulphacetamide

m.p. 181° to 184° C. Molecular weight 214.
*Phthalylsulphthiazole*, molecular weight 403.



phthalylsulphthiazole

*Sulphatriad* is a mixture of sulphadiazine, sulphathiazole, and sulphamerazine in such proportions that each tablet contains 0·185 gram, 0·185 gram, and 0·130 gram respectively.

## Determination in Blood

**Method of Bratton and Marshall** (1939). Micro-modification.
After precipitating the proteins with trichloracetic acid the sulphonamide present in the filtrate is diazotized and then coupled with N (1-naphthyl) ethylenediamine dihydrochloride and estimated colorimetrically.

**Reagents.** 1. Trichloracetic acid, 20 per cent. (w/v) solution.

2. Sodium nitrite, 0·1 per cent. solution. Prepare freshly at frequent intervals.

3. Ammonium sulphamate, 0·5 per cent. solution.

4. N (1-naphthyl) ethylenediamine dihydrochloride, 0·1 per cent. aqueous solution. Keep in a dark coloured bottle.

5. Hydrochloric acid, 4 N.

6. Stock standard solution of the sulphonamide containing 10 mg. per 100 ml. Dissolve 100 mg. in water and make up to 1 litre. Whilst it is most satisfactory to use the actual sulphonamide which is being determined, a solution of any one of them can be used and the necessary correction made for the difference in molecular weight. Sulphanilamide has been frequently used but the molecular weights of sulphapyridine, sulphadiazine and sulphathiazole are near to each other and to other commonly used ones, so that one of these is perhaps more suitable.

7. Standard solution for use. Add 20 ml. of 20 per cent. trichloracetic acid to 5 ml. of the stock standard and make up to 100 ml. with water. This contains 0·005 mg. per ml.

**Technique.** Pipette 3 ml. of water (or 0·05 per cent. saponin solution) into a test tube and add to it 0·2 ml. of blood which can be taken from a finger or thumb in a 0·2 pipette such as is used for blood sugar estimation. Then add 0·8 ml. of 20 per cent. trichloracetic acid, mix well and allow to stand for a few minutes. Filter through a 7 cm. No. 1 Whatman filter paper, or centrifuge. Pipette 2 ml. of the filtrate (= 0·1 ml. blood) into a suitable test tube and add 0·2 ml. of 0·1 per cent. sodium nitrite. Shake well and allow to stand for three minutes. Then add 0·2 ml. of 0·5 per cent. ammonium sulphamate, again mix and stand for two minutes. This removes excess of sodium nitrite. Finally add 1 ml. of the coupling agent. Allow to stand for ten minutes and compare with 2 ml. of the above standard similarly treated. Put up a blank containing water. With the photoelectric colorimeter use a yellow-green filter, transmission at 540 millimicrons.

**Calculation.** Using the photoelectric colorimeter.

Mg. sulphonamide per 100 ml. blood

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0{\cdot}010 \times \frac{100}{0{\cdot}1}$$

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 10.$$

If desired, a standard curve can be prepared by making up a series of standards using various amounts of stock standard, for example 0, 1·25, 2·5, 5, 7·5, 10 ml. added to 20 ml. trichloracetic acid and made up to 100 ml. These correspond respectively to 0, 2·5, 5, 10, 15, 20 mg. per 100 ml. blood.

Should a sulphonamide be used as standard other than that which is being determined, the following conversion table will be found useful.

| Standard | M. Wt. | Compound being determined | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Sulphanilamide | Sulphaguanidine | Sulphapyridine | Sulphadiazine | Sulphathiazole | Sulphamerazine | Succinylsulphathiazole | Sulphamethazine |
| Sulphanilamide . . | 172 | 1·00 | 1·24 | 1·45 | 1·45 | 1·48 | 1·55 | 2·06 | 1·62 |
| Sulphapyridine . . | 249 | 0·69 | 0·86 | 1·00 | 1·00 | 1·02 | 1·06 | 1·43 | 1·12 |
| Sulphadiazine . . | 250 | 0·69 | 0·86 | 1·00 | 1·00 | 1·02 | 1·06 | 1·42 | 1·11 |
| Sulphamezathine . | 278 | 0·62 | 0·77 | 0·90 | 0·90 | 0·92 | 0·95 | 1·28 | 1·00 |
| Sulphamerazine . . | 266 | 0·65 | 0·81 | 0·95 | 0·95 | 0·97 | 1·00 | 1·35 | 1·05 |

(*From "The Estimation of Sulphonamides." May and Baker Ltd.*)

The method given above estimates the free drug. A certain amount is present as the acetyl derivative. However, only the free sulphonamide is active so that it alone needs to be determined. Total sulphonamide can be estimated by heating some of the filtrate after precipitating the proteins, with hydrochloric acid before carrying out the diazotization. Heat 2 ml. of such filtrate with 0·2 ml. of 4 N hydrochloric acid in boiling water for one hour. Cool and make up to 2 ml. with water. Proceed as before.

**Notes.** 1. Urea may be used instead of ammonium sulphamate for removing the excess nitrite. A 0·5 per cent. solution is satisfactory. Other coupling agents have also been used, for example, a 0·4 per cent. alcoholic solution of dimethyl α-naphthylamine. This should be kept in the dark.

2. The same method may be used on other fluids, for example, on cerebrospinal fluid. Requests for estimation in this are sometimes made in cases of meningitis and cerebral abscess. Except when the blood level is falling, the concentration in the cerebrospinal fluid is usually about 50 to 70 per cent. of that in the blood unless the drug is given into the theca.

3. Determination of sulphonamides in urine may also be done in the same way but dilution is necessary. A dilution between 1 in 10 and 1 in 50 is usually required.

4. The above method may be used for derivatives of sulphanilamide in which substitution is into the sulphonamide group, as in sulphapyridine, sulphadiazine, sulphaguanidine, etc. When, however, substitution is in the amino group, it may be necessary to set free this group by acid hydrolysis before estimation can be carried out.

INTERPRETATION

Estimation of sulphonamides is not required frequently now that standardized methods of treatment have been worked out and other chemotherapeutic substances are available. However, they are still required on occasion and the method should be available. The levels aimed at in blood are usually in the region of 10 to 15 mg. per 100 ml. but some workers in the case of some cerebral lesions have

used much higher levels and aimed at and reached 30 to 40 mg. per 100 ml. For blood levels of more than 20 mg. per 100 ml. it is better to use 0·1 ml. of blood.

## THIOCYANATES

**Determination of Serum Thiocyanates** (Bowler, 1944)

After precipitating the proteins with trichloracetic acid, thiocyanates are estimated colorimetrically as ferric thiocyanate after addition of ferric nitrate. Bowler studied the conditions necessary for optimum colour development and found that ferric nitrate should be present in large excess and that the final solution should be 0·5 N nitric acid. Furthermore, reading of the colour should take place within fifteen minutes and the coloured solution should not be exposed to bright light.

**Reagents.** 1. Trichloracetic acid, 20 per cent. solution. Dissolve 20 grams in water, make to 100 ml., and filter.

2. Ferric nitrate-nitric acid reagent. Dissolve 80 grams of ferric nitrate, $Fe(NO_3)_3 . 9H_2O$, in 250 ml. of 2 N nitric acid, make to 500 ml. with water and filter. Concentrated nitric acid is approximately 16 N so that 2 N acid contains very nearly 31·5 ml. of concentrated acid in 250 ml. Keep in the dark.

3. Standard thiocyanate solution. Prepare a standard solution containing 10 mg. SCN per 100 ml. of solution, by diluting 1 to 100 with water a thiocyanate solution containing 1 gram SCN per 100 ml. Standardize against silver nitrate solution. Twenty ml. of N/10 silver nitrate would require 11·6 ml. of the thiocyanate solution. Or, if N/10 thiocyanate is kept in the laboratory, the standard thiocyanate (10 mg. per 100 ml.) can be prepared by diluting 1 ml. of this to 58 ml. with water.

**Technique.** Pipette 1 ml. of serum into a test tube, add 6·5 ml. of water and 2·5 ml. of 20 per cent. trichloracetic acid. Mix well and allow to stand for ten to fifteen minutes. Filter through a Whatman No. 40 filter paper (9 cm.). To 5 ml. of the filtrate add 5 ml. of ferric nitrate reagent, and keeping out of bright light, compare with a standard prepared at the same time or read on the photoelectric colorimeter using a blue filter or transmission at 470 millimicrons. Prepare the standard by pipetting 1 ml. normal serum into a test tube and then add 5·5 ml. water, 1 ml. of thiocyanate standard and 2·5 ml. of trichloracetic acid. Mix well and proceed as with the test.

**Calculation.** Since a 10 mg. per 100 ml. solution is put through in exactly the same way as the serum, the calculation is simple. For the visual colorimeter,

Mg. thiocyanate per 100 ml. serum

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 10$$

the results being as SCN.

For the photoelectric colorimeter a standard curve can be prepared as follows, using a solution containing 10 mg. per 100 ml.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mg. SCN per 100 ml. serum | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 16 |
| Normal Serum (free from SCN) | Add 1 ml. to each. | | | | | | | |
| Ml. thiocyanate standard | 0 | 0·2 | 0·4 | 0·6 | 0·8 | 1·0 | 1·2 | 1·6 |
| Ml. water | 6·5 | 6·3 | 6·1 | 5·9 | 5·7 | 5·5 | 5·3 | 4·9 |

Add 2·5 ml. of trichloracetic acid to each, mix, filter, add 5 ml. ferric nitrate solution to 5 ml. filtrate in each case and read as for the test.

**Note.** The determination must be carried out on serum. Both oxalate and citrate produce a colour with the ferric nitrate.

INTERPRETATION

Thiocyanate has been given as a treatment for hypertension. The dosage recommended is that which will produce a serum thiocyanate of about 10 mg. per 100 ml. Thiocyanate, however, may produce toxic symptoms at serum levels above about 12 mg. per cent. so careful control is necessary. The value of thiocyanate in hypertension would appear doubtful.

## LEAD

### Determination in Urine

The method given is essentially that of Kench (1940) using wet oxidation of organic matter followed by estimation of the lead as a complex with dithizone after extraction with sodium diethyldithiocarbamate.

**Reagents.** 1. Sulphuric acid, 2 N. This should be deleaded as follows. Dilute some concentrated sulphuric acid 1 in 20 and shake with 20 to 30 mg. of sodium diethyldithiocarbamate three times, using ether containing 5 per cent. carbon tetrachloride the first two occasions and ether only on the third. Then carefully evaporate off the ether by heating on a water bath in a current of air, or better, on an electric hot plate, well away from flames.

2. Sodium citrate, 20 per cent. solution, deleaded in the same way by shaking with ether and sodium diethyldithiocarbamate.

3. Potassium cyanide, 10 per cent. solution. British Drug Houses Pb T is satisfactory.

4. Potassium cyanide, 1 per cent. solution. Dilute the above 10 per cent. solution with distilled water redistilled from glass.

5. Ammonia, S.G. 0·88. Use high grade analytical reagent quality.

6. Hydrogen peroxide, 100 vols.

7. Ether, of high grade analytical reagent quality.

8. Carbon tetrachloride, of the same quality.

9. Powdered sodium diethyldithiocarbamate.

10. Dithizone. Prepare a solution for use as follows. Dissolve about 20 mg. of dithizone in about 10 ml. of carbon tetrachloride or chloroform and extract this with about 10 ml. of 5 per cent. ammonia. Reprecipitate the dithizone by adding N hydrochloric acid to the ammonia extract and then take up again in a few ml. of carbon tetrachloride. Finally, just before use, extract the dithizone thus purified with about 5 ml. of 5 per cent. ammonia. This is ready for use.

11. Glacial acetic acid. High grade analytical reagent.

12. Standard lead solution. Prepare a stock solution by dissolving 185 mg. of lead acetate $(CH_3CO.O)_2Pb.3H_2O$, in water to which has been added a little glacial acetic acid and then make up to 100 ml. This solution contains 1 mg. of lead per ml. For use in the test dilute 1 ml. to 100 ml. with redistilled water. The solution now contains 10 $\mu$g. per ml.

**Technique.** Evaporate almost to fuming about 150 ml. of the deleaded 2 N sulphuric acid in a 300 ml. Pyrex Kjeldahl flask using small pieces of Pyrex rod to prevent bumping. It should be noted that some glass beads contain lead. Depending on the specific gravity of the urine add 40 to 100 ml. of the urine slowly to this concentrated acid, using 40 ml. with urines of high specific gravity and 100 ml. with very dilute urines and intermediate amounts with other urines. The amount to use can be assessed from the appearance of the urine. Fifty ml. is satisfactory for normal twenty-four-hour specimens. Heat the mixture carefully. If it tends to froth, as it often does when the greater part of the water has been driven off, reduce to a very small flame until this tendency stops. Then continue heating gently until the colour clears almost to colourless. This may take one to three hours. A few drops of hydrogen peroxide can be used to hasten this process if desired. If this is done, cool, add the hydrogen peroxide and then gently heat again.

When oxidation of organic matter is complete, cool, and add carefully 20 to 30 ml. of distilled water, redistilled from glass, and make just alkaline to litmus with ammonia. Add 5 ml. of 20 per cent. citrate. Transfer with washing to a Pyrex separating funnel and add 5 ml. of 10 per cent. potassium cyanide. Extract twice by shaking with 20 to 30 ml. of ether and 20 to 30 mg. of powdered sodium diethyldithiocarbamate. Run the extracts into a 6 × 1 inch Pyrex test tube and evaporate off the ether. This is most conveniently done by allowing the ether to evaporate off spontaneously by placing the tubes in a warm place away from flames. Keep the tubes covered with filter paper.

To the residue add 0·4 ml. of concentrated sulphuric acid obtained by reconcentrating the deleaded 2 N acid and destroy organic matter by heating gently until clear and colourless. Add 4 ml. of distilled water, redistilled from glass, 0·2 ml. of glacial acetic acid, 2·2 ml. of ammonia, transfer to a small separating funnel and wash out the test tube with 5 ml. of 1 per cent. potassium cyanide adding this to the separating funnel. Then add 5 ml. of carbon tetrachloride and 3 drops of the solution of purified dithizone. The red coloured compound of dithizone with lead is extracted into the carbon tetrachloride layer, leaving the excess of dithizone in the aqueous ammonia. Run off the carbon tetrachloride layer and shake with a few ml. of 1 per cent. cyanide to remove any excess dithizone. This usually has no appreciable effect and may be omitted.

Put up a series of standards containing 0, 2·5, 5, 7·5, 10 $\mu$g. of lead, that is using 0, 0·25, 0·5, 0·75, 1 ml. of the diluted standard and adding 2 drops of concentrated sulphuric acid, 4 ml. of water,

ammonia, acetic acid, and so on in the same way as for the test, which is then matched against the appropriate standard, or a standard curve prepared for the photoelectric colorimeter in which case a yellow-green filter, transmission at 540 millimicrons is employed. Remove traces of water by shaking with a little anhydrous sodium sulphate. Use the 0 specimen to set the colorimeter for the standards.

Carry out a complete blank omitting only the urine. This is usually about 1 to 1·5 μg. and should be subtracted from the reading of the test. This can be used to set the colorimeter for reading the unknowns.

**Calculation.** If a twenty-four-hour specimen of urine is being used the lead excretion can be easily calculated.

Daily excretion in μg.

$$= \mu g. \text{ in the unknown} \times \frac{\text{ml. of twenty-four hour specimen}}{\text{ml. of urine taken for the test}}$$

It may, however, not be convenient to collect a twenty-four-hour specimen in which case a single specimen of urine can be passed directly into a Pyrex flask, the specific gravity of such a specimen determined, and a correction applied for variations in concentration of the urine by referring to a specific gravity of 1·026 and expressing the result as micrograms of lead per litre of urine of this specific gravity. For example, 50 ml. of urine were found to contain 4·8 μg. of lead, the blank being 1·4 μg. The specific gravity was 1·022. Then

$$\text{Corrected excretion} = (4{\cdot}8 - 1{\cdot}4) \times \frac{1{,}000}{50} \times \frac{1{\cdot}026}{1{\cdot}022} \mu g. \text{ per litre.}$$
$$= 80 \mu g. \text{ per litre.}$$

**Note.** The author agrees with Kench in preferring wet oxidation of the organic matter, but other workers have chosen dry ashing. Thus Tompsett and Anderson (1935) use ashing in a silica basin. Otherwise their method is roughly similar to the above though differing in details.

INTERPRETATION

The normal amount of lead excreted may be taken as being between 30 and 80 μg. daily or about the same amount per litre corrected to a specific gravity of 1·026. It will naturally be influenced by the amount of lead in the drinking water. In the author's experience in the Manchester district the excretion in normal persons is within fairly narrow limits averaging about 40 μg. daily.

The amount excreted in acute lead poisoning may be increased up to about 300 μg. daily, but returns to normal limits in a few weeks' time if the person is no longer exposed to lead (see Kench *et al.*). So, if there is no recent exposure to lead, urine lead is not often significantly increased. It has been suggested that blood levels may be more informative.

The test is of considerable value in assessing the hazard to which workers are exposed in certain industries using lead and lead com-

pounds. In such cases considerable amounts of lead are excreted, sometimes exceeding 200 μg. daily. The collection of single specimens from a batch of workers engaged on a certain process may thus be used. An average value for the lead excretion of such a group gives a good indication of the amount of lead which is being absorbed. It is seen that almost as much may be found in the urine of lead workers who show no symptoms of lead poisoning as in the urine of patients actually suffering from acute lead poisoning.

### Excretion of Lead in the Fæces

A much greater amount of lead is present in the fæces than in the urine. This is lead which has not been absorbed in the intestine. A commonly accepted average figure is 0·3 mg. daily, given by Kehoe *et al.* (1933), and Tompsett and Anderson (1935), who give 0·22 to 0·4 mg. daily. There is, however, considerable variation and it has been suggested that amounts up to 0·6 mg. and even up to 1 mg., may be reached before lead poisoning can be held to be present (see Kehoe ; Tompsett and Anderson, 1939).

#### Determination in Fæces

Ashing of the fæces is desirable. Dry the fæces, powder and weigh out about 1 gram in a small basin and ash in the electric furnace at about 400° C. Dissolve in about 100 ml. of the deleaded 2 N sulphuric acid and proceed as described for the estimation in urine.

### Lead in Blood

The amount of lead in blood has been held to give the best indication as to whether there is lead poisoning, but the estimation is not so easily carried out as with urine.

**Estimation.** Use 20 ml. of citrated blood and proceed in the same way as described for urine, using hydrogen peroxide to complete the digestion.

INTERPRETATION

The normal concentration in the blood is in the range 10 to 30 μg. per 100 ml. Litzner and Wehrmeh (1933) state that symptoms of poisoning may begin at 30 μg. per cent. with severe symptoms always present above 60 to 70, but other workers have reported up to 100 μg. per 100 ml. without symptoms (Tompsett and Anderson, 1939). The author's somewhat limited experience agrees with the above range for normals. See also Willoughby and Wilkins (1938) and Monier-Williams (1949), who gives a valuable survey of the distribution of lead in the body and in foods, and of the methods used in its determination.

## MERCURY

#### Determination in Urine

Organic matter is destroyed by means of potassium permanganate the excess of which is removed by adding hydroxylamine hydrochloride. The mercury is then extracted with dithizone and

estimated colorimetrically. The method as given is derived from the papers of Hubbard (1940) and of Milton and Hoskins (1947).

**Reagents.** 1. Sulphuric acid. Add 1 volume of concentrated acid carefully to 2 volumes of water.

2. Solid potassium permanganate.

3. Hydroxylamine hydrochloride. Five per cent. aqueous solution.

4. Stock dithizone solution. One hundred mg. per 100 ml. of chloroform. Purify the dithizone as in the case of lead estimation, that is, dissolve in chloroform and extract from this with 5 per cent. ammonia, precipitate with weak hydrochloric acid from the ammonia extract, take up in chloroform, evaporate off the chloroform and dry in an incubator.

5. Dithizone solution for use. Dilute 5 ml. of stock solution to 100 ml. of chloroform. This solution contains 5 mg. dithizone per 100 ml.

6. Stock mercuric chloride solution containing 0·135 gram per 100 ml. of solution. This contains 1 mg. mercury per ml.

7. Dilute mercuric chloride solution. Dilute 1 ml. of stock solution to 100 ml. so that 1 ml. contains 10 $\mu$g. of mercury.

**Technique.** Measure 50 ml. of urine into a 200 ml. round flask fitted either with a cold finger condenser or a reflux condenser. Considerable care has to be taken during the oxidation of the organic matter to avoid loss of mercury by volatilization, hence the use of the condensers and only moderate heat. Add 10 ml. of sulphuric acid and about a third of a gram of potassium permanganate. Heat gently on a water bath. Continue adding further portions of permanganate at intervals until the colour of the permanganate persists. Cool and add hydroxylamine hydrochloride, drop by drop, until the solution is decolorized. Add 2 ml. excess, heat to boiling for a minute and cool. Transfer quantitatively to a separating funnel and extract three times with 5 ml. of the weak dithizone solution. Make up the combined dithizone extracts to 15 ml. with further weak dithizone solution.

The only metal which is likely to be present in urine and combine with dithizone at this very acid *p*H is copper. Hubbard extracted the mercury into an aqueous phase with sodium thiosulphate but Milton and Hoskins state that selective extraction of mercury by dilute thizone as described above is possible and give examples of solutions containing mercury and copper, with the latter in excess, so extracted without interference from the copper. The author has confirmed this.

Carry through a complete blank on the reagents, and prepare for the photoelectric colorimeter a series of standards from the weak mercuric chloride solution by extracting solutions containing 0, 10, 20, 30, 40 and 50 $\mu$g. of mercury in the same way as the extraction of the test.

The colour due to the mercury dithizone can be estimated as follows :

(*a*) Using a blue-green filter, transmission at 480 millimicrons,

and a double-ended colorimeter, place the test on one side and the unchanged weak dithizone solution on the other. In this way, the colours from the series of standards are also read, a curve prepared and the value of the test read from this. The difference between test and blank gives the amount of mercury present in the volume of urine taken.

(*b*) The above method may not be suitable for some colorimeters. Milton and Hoskins shake the dithizone extract twice with 10 ml. of 5 per cent. ammonia to remove the excess dithizone. The mercury dithizonate remains in the chloroform layer. Shake with anhydrous sodium sulphate, filter, and read in the colorimeter using a blue-green filter, transmission at 480 millimicrons. Prepare a standard curve from the above standards similarly treated.

INTERPRETATION

Estimation of mercury in the urine has been used in connection with acrodynia (pink disease) in infants. Teething powders containing calomel are the source. In normal urines up to about 8 $\mu$g. per 100 ml. of urine may be found. In some infants with pink disease amounts up to about 200 $\mu$g. per 100 ml. have been found. There is not general agreement as regards the part mercury may play in the ætiology of this condition (see Warkany and Hubbard, 1948, 1951).

The estimation is also of value occasionally in connection with workers exposed to mercury in industrial processes. Such workers may excrete as much as a milligram and over in a day. See, for example, Buckett *et al.* (1946), who found a daily excretion of 5 to 90 $\mu$g. in normal controls and up to 2,360 $\mu$g. in workers exposed to mercury.

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# APPENDICES

## UNITS

| | | | |
|---|---|---|---|
| 1 cm. . . | = 0·3937 in. | 1 in. . . | = 2·540 cm. |
| 1 metre . | = 3·281 ft. | 1 ft. . . | = 30·48 cm. |
| 1 kilometre. | = 1093·6 yd. | 1 yd. . . | = 0·9144 metre. |
| 1 sq. cm. . | = 0·155 sq. in. | 1 sq. in. . | = 6·452 sq. cm. |
| 1 sq. metre. | = 10·764 sq. ft. | 1 sq. ft. . | = 0·0926 sq. metre. |
| | | 1 sq. yd. . | = 0·836 sq. metre. |
| 1 cu. cm. . | = 0·0610 cu. in. | 1 cu. metre | = 1·308 cu. yd. |
| 1 cu. in. . | = 16·39 millilitres. | 1 cu. ft. . | = 28·32 litres. |
| 1 cu. yd. . | = 0·765 cu. metres. | | |
| 1 pint = 568·25 ml. = 20 fl. oz. | | 1 qt. . | = 1·136 litres. |
| 1 gall. . | = 4·546 litres. | 1 litre . | = 1·760 pints. |
| 1 gram . | = 15·43 grains. | 1 oz. . | = 28·35 grams. |
| 1 kilogram . | = 2·205 lb. | 1 lb. . . | = 453·6 grams. |

Note that the primary unit of volume, the litre, is defined as the volume of a kilogram of pure water at 4° C. 760 mm. Hg. One millilitre is a thousandth part of a litre. This is almost, but not quite, the same as a cubic centimetre, the relation being

1,000 ml. = 1000·027 c.c.

The cubic centimetre is derived from the standard unit of length, the metre. A cubic centimetre of water was intended to weigh a gram, but more accurate measurements of the standards adopted have shown that there is the slight difference stated above.

## STANDARDS FOR VOLUMETRIC ANALYSIS

The following "Analar" chemicals are recommended by British Drug Houses for use as primary standards. They are anhydrous salts and may be dried by heat without change of composition. The amounts given are for N/10 solutions.

| | |
|---|---|
| Sodium carbonate (anhydrous) . | 5·300 grams per litre. |
| Sodium oxalate . . . . | 6·70 ,, ,, |
| Sodium chloride . . . . | 5·845 ,, ,, |
| Potassium iodate . . . . | 3·567 ,, ,, |
| Potassium dichromate . . . | 4·904 ,, ,, |

As secondary standards the following may be used. These are liable to gain or lose moisture in accordance with the humidity of the atmosphere and the care with which they are stored.

| | |
|---|---|
| Ferrous ammonium sulphate . | 39·21 grams $FeSO_4(NH_4)_2SO_4$, $6H_2O$ per litre. |
| Oxalic acid . . . . | 6·3025 grams $(COOH)_2 2H_2O$ per litre. |
| Silver nitrate . . . | 16·989 grams $AgNO_3$ per litre. |

| | |
|---|---|
| Potassium hydrogen phthalate (for alkalies). | 20·41 grams $COOH.C_6H_4COOK$ per litre. |
| Antimony potassium tartrate (for iodine). | 16·245 grams, $KSbOC_4H_4O_6$ per litre. |
| Sodium borate (for hydrochloric acid). | 19·07 grams, $Na_2B_4O_7, 10H_2O$ per litre. |

British Drug Houses have now available ampoules of concentrated volumetric solutions, containing approximately 50 ml. of solution, which when diluted to 500 ml. with distilled water, give an accurately standardized solution. These cover all the commonly used volumetric solutions and should be useful for small laboratories. They are sold in cartons containing seven ampoules.

## INDICATORS

| | *p*H range | Colour change | |
|---|---|---|---|
| Thymol blue (acid range). | 1·2– 2·8 | Red to yellow. | 0·1 gm., 4·3 ml. N/20 NaOH, to 250 ml. with water. |
| Topfer's reagent . | 2·9– 4·2 | Red to yellow. | 0·5 gm. in 100 ml. 95 per cent. alcohol. |
| Bromphenol blue . | 3·0– 4·6 | Yellow to blue. | 0·1 gm., 3 ml. N/20 NaOH, to 250 ml. with water. |
| Methyl orange . | 3·0– 4·4 | Red to yellow. | 0·1 gm. in 100 ml. water. |
| Congo red . . | 3·0– 4·5 | Blue to red. | 0·5 gm., 90 ml. water, 10 ml. alcohol. |
| Bromcresol green . | 3·8– 5·4 | Yellow to green. | 0·1 gm., 2·9 ml. N/20 NaOH, to 250 ml. with water. |
| Methyl red . . | 4·4– 6·2 | Red to yellow. | 0·05 gm. in 100 ml. 50 per cent. alcohol. |
| Chlorphenol red . | 5·0– 6·6 | Yellow to red. | 0·1 gm., 4·8 ml. N/20 NaOH, to 250 ml. with water. |
| Bromcresol purple . | 5·2– 6·8 | Yellow to purple. | 0·1 gm., 3·7 ml. N/20 NaOH, to 250 ml. with water. |
| Litmus . . | 4·5– 8·3 | Red to blue. | |
| Bromthymol blue . | 6·0– 7·6 | Yellow to blue. | 0·1 gm., 3·2 ml. N/20 NaOH, to 250 ml. with water. |
| Phenol red . . | 6·8– 8·4 | Yellow to red. | 0·1 gm., 5·7 ml. N/20 NaOH, to 250 ml. with water. |
| Cresol red . . | 7·2– 8·8 | Yellow to red. | 0·1 gm., 5·3 ml. N/20 NaOH, to 250 ml. with water. |
| Thymol blue (alkaline range). | 8·0– 9·6 | Yellow to blue. | As above. |
| Phenolphthalein . | 8·3–10·0 | Colourless to red. | 0·1 to 1 per cent. in 50 per cent. alcohol. |
| Thymolphthalein . | 9·3–10·5 | Colourless to blue. | 0·1 per cent. in alcohol. |
| Alizarin yellow 9 . | 10·1–12·1 | Colourless to yellow. | 0·1 per cent. in water. |
| Tropæolin O . . | 11·1–12·7 | Yellow to orange. | 0·1 per cent. in water. |

If stronger solutions are required, make up to 100 ml. instead of 250 ml. where stated.

## BUFFER MIXTURES

The following mixtures, which cover the range from *p*H 1·2 to 10·0, are those of Clark. ("Determination of Hydrogen Ions," 3rd ed., Bailliere, Tindall and Cox, London.)

I. *KCl–HCl Mixtures* (at 20° C.)

Ml. M/5 HCl to be added to 50 ml. M/5 KCl and diluted to 200 ml. :

| *p*H | ml. M/5 HCl | *p*H | ml. M/5 HCl | *p*H | ml. M/5 HCl |
|---|---|---|---|---|---|
| 1·2 | 65·5 | 1·6 | 26·3 | 2·0 | 10·6 |
| 1·4 | 41·5 | 1·8 | 16·6 | 2·2 | 6·7 |

II. *Phthalate–HCl Mixtures* (at 20° C.)

Ml. M/5 HCl to be added to 50 ml. of M/5 potassium hydrogen phthalate and diluted to 200 ml. :

| *p*H | ml. M/5 HCl | *p*H | ml. M/5 HCl | *p*H | ml. M/5 HCl |
|---|---|---|---|---|---|
| 2·2 | 46·60 | 2·8 | 26·50 | 3·4 | 9·95 |
| 2·4 | 39·60 | 3·0 | 20·40 | 3·6 | 6·00 |
| 2·6 | 33·00 | 3·2 | 14·80 | 3·8 | 2·65 |

III. *Phthalate–NaOH Mixtures* (at 20° C.)

Ml. M/5 NaOH to be added to 50 ml. of M/5 potassium hydrogen phthalate and diluted to 200 ml.

| *p*H | ml. M/5 NaOH | *p*H | ml. M/5 NaOH | *p*H | ml. M/5 NaOH |
|---|---|---|---|---|---|
| 4·0 | 0·40 | 4·8 | 17·50 | 5·6 | 39·70 |
| 4·2 | 3·65 | 5·0 | 23·65 | 5·8 | 43·10 |
| 4·4 | 7·35 | 5·2 | 29·75 | 6·0 | 45·40 |
| 4·6 | 12·00 | 5·4 | 35·25 | 6·2 | 47·00 |

IV. *Potassium Dihydrogen Phosphate–NaOH Mixtures* (at 20° C.)

Ml. M/5 NaOH to be added to 50 ml. of M/5$KH_2PO_4$ and diluted to 200 ml.

| *p*H | ml. M/5 NaOH | *p*H | ml. M/5 NaOH | *p*H | ml. M/5 NaOH |
|---|---|---|---|---|---|
| 5·8 | 3·66 | 6·6 | 17·74 | 7·4 | 39·34 |
| 6·0 | 5·64 | 6·8 | 23·60 | 7·6 | 42·74 |
| 6·2 | 8·55 | 7·0 | 29·54 | 7·8 | 45·17 |
| 6·4 | 12·60 | 7·2 | 34·90 | 8·0 | 46·85 |

V. *Boric Acid, KCl–NaOH Mixtures* (at 20° C.)

Ml. M/5 NaOH to be added to 50 ml. of M/5 $H_3BO_3$-M/5 KCl and diluted to 200 ml.

| *p*H | ml. M/5 NaOH | *p*H | ml. M/5 NaOH | *p*H | ml. M/5 NaOH |
|---|---|---|---|---|---|
| 7·8 | 2·65 | 8·6 | 12·00 | 9·4 | 32·00 |
| 8·0 | 4·00 | 8·8 | 16·40 | 9·6 | 36·85 |
| 8·2 | 5·90 | 9·0 | 21·40 | 9·8 | 40·80 |
| 8·4 | 8·55 | 9·2 | 26·70 | 10·0 | 43·90 |

Sörenson has used a different set of buffer mixtures, some of which are as follows.

VI. *Mixtures of M/15 $KH_2PO_4$ and M/15 $Na_2HPO_4$*

M/15 potassium dihydrogen phosphate, 9·078 grams $KH_2PO_4$ per litre of solution.

M/15 disodium hydrogen phosphate, 11·876 grams $Na_2HPO_4$, $2H_2O$ (Sörenson's salt) per litre of solution.

| *p*H | ml. M/15 $Na_2HPO_4$ | ml. M/15 $KH_2PO_4$ | *p*H | ml. M/15 $Na_2HPO_4$ | ml. M/15 $KH_2PO_4$ |
|---|---|---|---|---|---|
| 5·8 | 8·0 | 92·0 | 7·10 | 66·6 | 33·4 |
| 5·9 | 9·9 | 90·1 | 7·15 | 69·2 | 30·8 |
| 6·0 | 12·2 | 87·8 | 7·20 | 72·0 | 28·0 |
| 6·1 | 15·3 | 84·7 | 7·25 | 74·4 | 25·6 |
| 6·2 | 18·6 | 81·4 | 7·30 | 76·8 | 23·2 |
| 6·3 | 22·4 | 77·6 | 7·35 | 78·9 | 21·1 |
| 6·4 | 26·7 | 73·3 | 7·40 | 80·8 | 19·2 |
| 6·5 | 31·8 | 68·2 | 7·45 | 82·5 | 17·5 |
| 6·6 | 37·5 | 62·5 | 7·50 | 84·1 | 15·9 |
| 6·7 | 43·5 | 56·5 | 7·55 | 85·7 | 14·3 |
| 6·8 | 49·6 | 50·4 | 7·60 | 87·0 | 13·0 |
| 6·85 | 52·5 | 47·5 | 7·65 | 88·2 | 11·8 |
| 6·90 | 55·4 | 44·6 | 7·70 | 89·4 | 10·6 |
| 6·95 | 58·2 | 41·8 | 7·75 | 90·5 | 9·5 |
| 7·00 | 61·1 | 38·9 | 7·80 | 91·5 | 8·5 |
| 7·05 | 63·9 | 36·1 | 7·9 | 93·2 | 6·8 |

VII. *Mixtures for pH range from 2·6 to 12·0* (Johnson and Lindsay, 1939, *Analyst*, **64**, 490)

Prepare a solution containing 6·008 grams of citric acid (A.R.), 3·893 grams of potassium dihydrogen phosphate (A.R.), 1·769 grams of boric acid (A.R.) and 5·266 grams of pure diethyl barbituric acid in a litre. The volumes of N/5 sodium hydroxide (carbonate-free) which are added to 100 ml. of the above solution to obtain different *p*H values are as follows :

| *p*H | ml. N/5 NaOH | *p*H | ml. N/5 NaOH | *p*H | ml. N/5 NaOH |
|---|---|---|---|---|---|
| 2·6 | 2·0 | 5·8 | 36·5 | 9·0 | 72·7 |
| 2·8 | 4·3 | 6·0 | 38·9 | 9·2 | 74·0 |
| 3·0 | 6·4 | 6·2 | 41·2 | 9·4 | 75·9 |
| 3·2 | 8·3 | 6·4 | 43·5 | 9·6 | 77·6 |
| 3·4 | 10·1 | 6·6 | 46·0 | 9·8 | 79·3 |
| 3·6 | 11·8 | 6·8 | 48·3 | 10·0 | 80·8 |
| 3·8 | 13·7 | 7·0 | 50·6 | 10·2 | 82·0 |
| 4·0 | 15·5 | 7·2 | 52·9 | 10·4 | 82·9 |
| 4·2 | 17·6 | 7·4 | 55·8 | 10·6 | 83·9 |
| 4·4 | 19·9 | 7·6 | 58·6 | 10·8 | 84·9 |
| 4·6 | 22·4 | 7·8 | 61·7 | 11·0 | 86·0 |
| 4·8 | 24·8 | 8·0 | 63·7 | 11·2 | 87·7 |
| 5·0 | 27·1 | 8·2 | 65·6 | 11·4 | 89·7 |
| 5·2 | 29·5 | 8·4 | 67·5 | 11·6 | 92·0 |
| 5·4 | 31·8 | 8·6 | 69·3 | 11·8 | 95·0 |
| 5·6 | 34·2 | 8·8 | 71·0 | 12·0 | 99·6 |

## BOILING POINTS

| | |
|---|---|
| Ethyl ether | 35° C. |
| Petroleum ether. Various fractions : | Below 40°, 40° to 60°, 50° to 60°, 60° to 80°, 80° to 100°, 100° to 120° ; above 120° C. |
| Light petroleum | 40° to 50°, 50° to 60° C. |
| Carbon disulphide | 46·2° |
| Acetone | 56·5° |
| Chloroform | 61° |
| Methyl alcohol | 66° |
| Ethyl acetate | 77° |
| Carbon tetrachloride | 78° |
| Ethyl alcohol | 78° |
| Benzene | 80° |
| Isobutyl alcohol | 106° |
| Toluene | 111° |
| Pyridine | 115° |
| *n*-butyl alcohol | 117° |
| Glacial acetic acid | 119° |
| Isoamyl alcohol | 131° |
| Xylene, *p*- | 137° |
| *m*- | 139° |
| *o*- | 142° |
| Isoamyl acetate | 139° |
| Aniline | 183° |
| Nitrobenzene | 210° |

## STANDARD WEIGHTS ACCORDING TO HEIGHT AND AGE

*(From "A Textbook of Dietetics," L. S. P. Davidson and I. A. Anderson. E. S. Livingstone)*

| Height | Age Period | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 15–19 | 20–24 | 25–29 | 30–34 | 35–40 | 41–44 | 45–49 | 50–54 | 55– |
| MEN : ft. in. | st. lb. | st. lb. | st. lb. | st. lb. | st. lb. | st. lb. | st. lb. | st. lb. | st. lb. |
| 4 11 | 7 13 | 8 3 | 8 10 | 8 13 | 9 1 | 9 4 | 9 6 | 9 7 | 9 8 |
| 5 0 | 8 1 | 8 7 | 8 12 | 9 1 | 9 3 | 9 6 | 9 8 | 9 9 | 9 10 |
| 5 1 | 8 3 | 8 9 | 9 0 | 9 3 | 9 5 | 9 8 | 9 10 | 9 11 | 9 12 |
| 5 2 | 8 6 | 8 12 | 9 2 | 9 5 | 9 7 | 9 10 | 9 12 | 9 13 | 10 0 |
| 5 3 | 8 9 | 9 1 | 9 5 | 9 8 | 9 10 | 9 13 | 10 1 | 10 2 | 10 3 |
| 5 4 | 8 12 | 9 5 | 9 8 | 9 11 | 10 0 | 10 2 | 10 4 | 10 5 | 10 6 |
| 5 5 | 9 2 | 9 9 | 9 12 | 10 1 | 10 4 | 10 6 | 10 8 | 10 9 | 10 10 |
| 5 6 | 9 6 | 9 13 | 10 2 | 10 5 | 10 8 | 10 10 | 10 12 | 10 13 | 11 0 |
| 5 7 | 9 10 | 10 2 | 10 6 | 10 9 | 10 12 | 11 0 | 11 2 | 11 3 | 11 4 |
| 5 8 | 10 0 | 10 6 | 10 10 | 11 0 | 11 3 | 11 5 | 11 7 | 11 8 | 11 9 |
| 5 9 | 10 4 | 10 10 | 11 0 | 11 4 | 11 8 | 11 10 | 11 12 | 11 13 | 12 0 |
| 5 10 | 10 8 | 11 0 | 11 4 | 11 9 | 11 13 | 12 1 | 12 3 | 12 4 | 12 5 |
| 5 11 | 10 13 | 11 4 | 11 9 | 12 0 | 12 4 | 12 7 | 12 9 | 12 10 | 12 11 |
| 6 0 | 11 4 | 11 9 | 12 1 | 12 6 | 12 10 | 12 13 | 13 1 | 13 2 | 13 3 |
| 6 1 | 11 9 | 12 0 | 12 7 | 12 12 | 13 2 | 13 5 | 13 8 | 13 9 | 13 10 |
| 6 2 | 12 0 | 12 5 | 12 13 | 13 4 | 13 9 | 13 12 | 14 1 | 14 2 | 14 3 |
| 6 3 | 12 5 | 12 10 | 13 5 | 13 10 | 14 1 | 14 5 | 14 8 | 14 9 | 14 10 |
| WOMEN | | | | | | | | | |
| 4 11 | 7 12 | 8 1 | 8 4 | 8 7 | 8 10 | 9 0 | 9 3 | 9 5 | 9 6 |
| 5 0 | 8 0 | 8 3 | 8 6 | 8 9 | 8 12 | 9 2 | 9 5 | 9 7 | 9 8 |
| 5 1 | 8 2 | 8 5 | 8 8 | 8 11 | 9 0 | 9 4 | 9 7 | 9 9 | 9 10 |
| 5 2 | 8 5 | 8 8 | 8 10 | 8 13 | 9 3 | 9 7 | 9 10 | 9 12 | 10 0 |
| 5 3 | 8 8 | 8 11 | 8 13 | 9 2 | 9 6 | 9 10 | 9 13 | 10 1 | 10 3 |
| 5 4 | 8 11 | 9 0 | 9 3 | 9 6 | 9 10 | 9 13 | 10 2 | 10 4 | 10 6 |
| 5 5 | 9 0 | 9 3 | 9 6 | 9 10 | 10 0 | 10 3 | 10 6 | 10 8 | 10 10 |
| 5 6 | 9 4 | 9 7 | 9 10 | 10 0 | 10 4 | 10 7 | 10 11 | 10 12 | 10 13 |
| 5 7 | 9 8 | 9 11 | 10 0 | 10 4 | 10 8 | 10 11 | 11 1 | 11 3 | 11 4 |
| 5 8 | 9 12 | 10 1 | 10 4 | 10 8 | 10 12 | 11 1 | 11 5 | 11 8 | 11 9 |
| 5 9 | 10 1 | 10 5 | 10 8 | 10 12 | 11 2 | 11 5 | 11 9 | 11 12 | 11 13 |
| 5 10 | 10 5 | 10 9 | 10 12 | 11 1 | 11 5 | 11 8 | 11 12 | 12 2 | 12 5 |
| 5 11 | 10 10 | 10 13 | 11 1 | 11 4 | 11 8 | 11 12 | 12 2 | 12 6 | 12 9 |
| 6 0 | 11 1 | 11 3 | 11 5 | 11 8 | 11 11 | 12 1 | 12 5 | 12 9 | 13 0 |

Height. Without shoes.
Weight. Indoor clothes without shoes.
For naked weight subtract 10 lb. for men, 6 lb. for women.

## ATOMIC WEIGHTS (1949)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Aluminium | Al | 13 | 26·97 | Molybdenum | Mo | 42 | 95·95 |
| Antimony | Sb | 51 | 121·76 | Neon | Ne | 10 | 20·183 |
| Argon | A | 18 | 39·944 | Nickel | Ni | 28 | 58·69 |
| Arsenic | As | 33 | 74·91 | Nitrogen | N | 7 | 14·008 |
| Barium | Ba | 56 | 137·36 | Osmium | Os | 76 | 190·2 |
| Beryllium | Be | 4 | 9·013 | Oxygen | O | 8 | 16·0000 |
| Bismuth | Bi | 83 | 209·00 | Palladium | Pd | 46 | 106·7 |
| Boron | B | 5 | 10·82 | Phosphorus | P | 15 | 30·98 |
| Bromine | Br | 35 | 79·916 | Platinum | Pt | 78 | 195·23 |
| Cadmium | Cd | 48 | 112·41 | Potassium | K | 19 | 39·096 |
| Cæsium | Cs | 55 | 132·91 | Radium | Ra | 88 | 226·05 |
| Calcium | Ca | 20 | 40·08 | Radon | Rn | 86 | 222 |
| Carbon | C | 6 | 12·01 | Rubidium | Rb | 37 | 85·48 |
| Cerium | Ce | 58 | 140·13 | Scandium | Sc | 21 | 45·10 |
| Chlorine | Cl | 17 | 35·457 | Selenium | Se | 34 | 78·96 |
| Chromium | Cr | 24 | 52·01 | Silicon | Si | 14 | 28·06 |
| Cobalt | Co | 27 | 58·94 | Silver | Ag | 47 | 107·880 |
| Copper | Cu | 29 | 63·54 | Sodium | Na | 11 | 22·997 |
| Fluorine | F | 9 | 19·000 | Strontium | Sr | 38 | 87·63 |
| Gallium | Ga | 31 | 69·72 | Sulphur | S | 16 | 32·066 |
| Gold | Au | 79 | 197·2 | Tantalum | Ta | 73 | 180·88 |
| Helium | He | 2 | 4·003 | Tellurium | Te | 52 | 127·61 |
| Hydrogen | H | 1 | 1·0080 | Thallium | Tl | 81 | 204·39 |
| Iodine | I | 53 | 126·92 | Thorium | Th | 90 | 232·12 |
| Iridium | Ir | 77 | 193·1 | Tin | Sn | 50 | 118·70 |
| Iron | Fe | 26 | 55·85 | Titanium | Ti | 22 | 47·90 |
| Krypton | Kr | 36 | 83·7 | Tungsten | W | 74 | 183·92 |
| Lead | Pb | 82 | 207·21 | Uranium | U | 92 | 238·07 |
| Lithium | Li | 3 | 6·940 | Vanadium | V | 23 | 50·95 |
| Magnesium | Mg | 12 | 24·32 | Xenon | Xe | 54 | 131·3 |
| Manganese | Mn | 25 | 54·93 | Zinc | Zn | 30 | 65·38 |
| Mercury | Hg | 80 | 200·61 | Zirconium | Zr | 40 | 91·22 |

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